

ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF NEUROLOGICAL AND COMMUNICATIVE DISORDERS AND STROKE
FISCAL YEAR 1980
VOLUME II

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service - National Institutes of Health

ANNUAL REPORT *of program activities*

October 1, 1979 through September 30, 1980

National Institute of Neurological and Communicative Disorders and Stroke
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Annual Report of the Scientific Director
of the
National Institute of Neurological and
Communicative Disorders and Stroke
October 1, 1979 through September 30, 1980

The mission of the NINCDS Intramural Research Program (IRP) is to advance, through the direct operation of its laboratories and clinics, new knowledge development in the neurosciences. Through studies ranging from basic neurobiologic probes to clinical trials of new therapeutic agents, IRP researchers during the past year have continued to make substantial progress towards elucidating the pathophysiologic bases for and therapeutic approaches to neurologic and communicative disorders. Indeed, as judged by the record of its scientific publications, IRP remains the world's foremost source of neuroscience research. Nevertheless, increasing administrative constraints have diminished the Program's ability to make optimal use of allocated resources and keep pace with newly emerging investigative opportunities.

It is especially in the field of personnel management that current governmental policies and procedures seem in greatest conflict with efficient operations and organizational needs. For example, allowable compensation and perquisite packages for scientists and physicians at mid and senior levels now lag substantially behind those offered by the universities, our chief competitors for research and patient care talent. Indeed, in most clinical areas, university entry salaries exceed those that are maximally attainable in Government service. The pay gap between Government and the private sector is of even more commanding proportions. Compounding this situation are the uncertainties and complexities of the newly introduced merit pay and Senior Executive Service (SES) systems, whose application of marginal monetary incentives to creative workers threatens to be counterproductive and divisive. Moreover, whatever theoretical advantages the SES system might confer to management are more than offset by the inflexibilities arising from its lack of convenient mechanisms for entry or exit and by the ceiling placed on the total number of SES positions available. Such matters have an unmistakable impact on the recruitment as well as the retention of able investigators; together with the increasing delays attending the appointment process, they have seriously eroded the Program's ability to compete for scientific talent. Largely for these reasons, efforts to obtain new leadership for the Laboratory of Neurophysiology and for the Positron Emission Tomography research program have remained unsuccessful.

Unpredictable shifts in personnel ceilings and prolonged prohibitions on hiring also continue to challenge program management. In February 1980 the IRP ceiling for full-time permanent positions was increased from 287 to 291. Utilization of these positions could have begun to address several critical needs within the Program. Unfortunately, however, the nearly simultaneous imposition of new restrictions on hiring frustrated this possibility. Thus, the last half of the fiscal year has witnessed a gradual decline in personnel strength so that by mid-September 1980, vacancies in nine authorized positions (mainly technical support and nontenured scientific categories) could not be filled. There is as yet no indication as to how long this hiring "freeze" might last.

Managerial efforts to deter the inevitable tendency towards increasing the proportion of tenured to nontenured scientific staff have continued. In fiscal year 1980 four tenured scientists left the Program. At the same time, a decision to recommend tenure was made for only one individual. Since 1975, the IRP has lost 12 tenured scientists in comparison with 20 gained by conversion or direct hiring to tenure status. Compensating for this increment in tenured staff (which numbered 68 during the current year) has been the increasing use of such non-ceiling (untentured) scientific categories as Guest Worker, Visiting Fellow, Expert, and Intergovernmental Personnel Act investigators. During FY 1980 the total number of doctoral level scientists (tenured and nontenured) averaged 248 in comparison with an average of 178 five years ago. Thus the Program has successfully maintained an essentially stable ratio of tenured to nontenured scientific staff.

Budgetary allocations in FY 1980 for direct and contract research were adequate to sustain IRP activities. The "other objects" apportionment for direct research operations totaled \$9.6 million, in comparison with \$9.2 million spent during the preceding year. Management of this allocation was complicated this year by the introduction of new constraints on fourth quarter spending and by mandating the inclusion of the cost of transporting things in the Program's total (heretofore exclusively personnel) travel ceiling. Contract research activity remained essentially constant during the past year, although the dollar amount fell to \$1.6 million, reflecting an earlier decision to "forward-fund" several major research contracts in order to facilitate the purchase of a cyclotron with FY 1980 funds. As in the past, IRP research contracts continued to provide important services (for example, primate holding or enzyme preparation) to facilitate high priority, in-house research.

Total space allocated to the Program also remained unchanged this year, thus perpetuating the inconveniences and inefficiencies inherent to a relatively crowded and widely dispersed facility. At present IRP occupies approximately 128,000 square feet of space, including 86,600 square feet on the main NIH campus (distributed between five buildings), 8,000 square feet off campus in the Bethesda area for laboratories (Parklawn Building) and offices (Federal Building), and 32,200 square feet in other locations (Fort Detrick Laboratories, Marine Biological Laboratories in Massachusetts, and Guam Memorial Hospital). The opening of the NIH Ambulatory Care Research Facility, now scheduled for late 1981, should markedly improve our situation. At that time, approximately 6,000 square feet of additional space will become available for Program use, and holdings now scattered throughout the NIH Clinical Center will be consolidated on the 4th and 5th floors.

Several changes in key staff occurring during the past year are worthy of special note, especially as they may adumbrate future directions for IRP-sponsored investigations. Dr. Susumo Sato of the Extramural Experimental Epilepsy Branch agreed to serve as Acting Chief of the Clinical Neurosciences Branch. Efforts to recruit permanent new leadership for IRP epilepsy research are continuing. Dr. Ronald Myers, Chief of the Section on Developmental Brain Pathology, resigned in July to assume new duties at the Cincinnati Veterans Administration Medical Center. His Section will be closed. Also during the past year all laboratory research conducted by the Laboratory of Experimental Neurology was phased out. Dr. King Engel, who for nearly 20 years directed the Program's neuromuscular disease research, announced his resignation effective July 1981. Part of the resources of the Medical Neurology Branch

will be re-allocated to support Positron Emission Tomography research. In this regard the Program's ORTEC ECAT Scanner is now in full operation for clinical studies. The limitations and delays associated with offsite isotope procurement has, however, prompted the decision to purchase a moderate energy cyclotron. Delivery is expected in about 2 years, at which time an appropriate facility can be built adjacent to the underground ACRF Parking Garage. A commitment to establish a new clinical program in communicative disorders has now led to the establishment of a search committee to seek appropriate leadership. Finally, plans have been initiated on Guam to move the NINCDS Clinical Research Center to a new building to be constructed on the grounds of the Guam Memorial Hospital.

By all criteria investigator-initiated research has continued to flourish within the Program. During the past year 13 projects were initiated, 32 were completed or terminated, and 117 projects remain active. The mix of this broad based effort remains essentially unchanged. The most extensively supported disciplines in the preclinical neurosciences are (in descending order): virology and immunology, physiology, chemistry, and pharmacology. Support of clinically applied investigations is now focused primarily on demyelinating disorders, metabolic-degenerative disorders, hearing, and tumors of the nervous system. There continues to be a roughly equal split between resource allocations for basic and clinically applied studies. Regarding the latter, 75 clinical protocols are now active, with an average of 25 patients being admitted to each study during the past year. Results of the Program's current research effort are presented in the appended Project Reports and in the Summaries provided by the Chiefs of each of the Program's 16 Laboratories and Branches.

Dissemination of IRP research findings continues to be worldwide. A total of 314 scientific articles were published during calendar year 1979. Journals in which these articles appeared in descending order of the number of IRP papers included: Archives of Neurology, Proceedings of the National Academy of Science, Advances in Neurology, Annals of Neurology, Journal of Biological Chemistry, Slow Transmissible Diseases of the Nervous System, Science, and Journal of Neurochemistry. In addition, IRP-authored papers appeared in foreign language journals from a number of foreign countries including France, Sweden, Japan, and the USSR. Program scientists also assisted in the rapid promulgation of new research findings by organizing and participating in meetings and workshops on a broad range of topics as well as through informal contacts with scientists from this country and abroad.

Another significant, yet sometimes overlooked, IRP contribution to neurosciences research is the superb training opportunities afforded scientists in many preclinical and clinical areas. Over the years a high proportion of IRP trainees have gone on to distinguished careers in academic and industrial research both here and in foreign countries. This year some 153 young investigators took advantage of training opportunities here as Staff Fellows (43), Clinical Associates (7), Visiting Fellows (46), Guest Workers (44), and Inter-governmental Personnel Act investigators (13). In addition, a clinical neurology training program for medical students has now been initiated. Moreover, the employment through the Equal Employment Opportunity summer program of undergraduate and graduate students is becoming an increasingly significant aspect of IRP training.

Finally, during the past year several IRP employees received special recognition for their outstanding contributions to neurosciences research. Dr. Roscoe Brady, DMNB, received the Cotzias Award from the American Academy of Neurology for his discovery of the cause, and development of procedures for the control, of lipid storage disorders. Dr. John Sever, IDB, won the Kimble Methodology Award for his work in developing novel diagnostic procedures and viral reagents now used worldwide. Dr. Thomas Reese, LNNS, was co-recipient of the W. Alden Spencer Award Medal from Columbia University. The Public Health Service's Special Recognition Award went to Dr. Monique Dubois-Dalcq, IDB, for her development of methods for labeling viruses in nerve cells, which have helped to improve our understanding of the pathogenesis of viral infections of the nervous system.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Instrumentation and Computers Section
National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report of Instrumentation and Computers Section

National Institute of Neurological and Communicative Disorders and Stroke

October 1, 1979 - September 30, 1980

The Instrumentation and Computers Section provides technical support for investigators by (1) assessing the instrumentation and computer needs of the investigator; (2) designing, developing and constructing special-purpose electronic and mechanical instrumentation and systems not commercially available; (3) designing, specifying and managing laboratory computer systems for data acquisition and processing.

Additional services provided by the Section include consultation on measurement techniques, signal processing, noise and electro-magnetic interference in data measurement systems, and equipment purchases. Several formal and informal courses for investigators are taught by Section personnel; topics include electrical circuit theory, operational amplifier applications, digital logic design, and computer applications.

Due to manpower limitations and economic considerations, the Section is unable to provide the following services: repair of commercial instruments, duplication of off-the-shelf commercially available equipment, and fabrication of non-instrument items (shelves, bookcases, etc.).

When an investigator requires the services of the Section, he first meets with the Section Chief and other personnel as needed to discuss his requirements. On the basis of this meeting, a decision is made as to whether ICS (Instrumentation and Computers Section) will take on the project. If a commercially produced instrument will satisfy the investigator's requirements, he is advised to purchase it. If custom instrumentation is needed, ICS will accept the project unless we lack the appropriate expertise, or our current work backlog is excessive. In these cases the project may be contracted to a private firm, or the investigator may be directed to the Biomedical Engineering and Instrumentation Branch (BEIB).

When the Section Chief or the Assistant to the Chief agree to accept a project, the investigator submits a standard work request form (available from ICS), signed by his Lab Chief. This form will state the nature of the instrument or service requested, and will contain as many details and specifications as the investigator can provide.

The project is then assigned to an engineer, who will confer with the investigator to formulate a set of engineering specifications and a timetable and cost estimate for the project. The ICS does not charge for services, but the investigator will be billed for the cost of the components used. Upon delivery of the completed instrument, a memo is sent to the investigator listing the component costs and asking permission to have the Administrative Officer transfer funds from his CAN to the Section's CAN.

INSTRUMENTATION

The following are selected instrumentation projects undertaken during the past year. These are chosen from a total of 288 projects, and are representative of the types of electronic instruments and systems developed by the Section:

(1) Patient Activity Monitoring System. The Section has continued to develop the NIMH Activity Monitor (PAM) and the ancillary hardware and computer software which forms the system. The major development of the past year has been the hybrid monitor. This device, developed under contract by a local thickfilm hybrid circuit company, features a memory with the capacity for 10 days of continuous monitoring at 15 minute resolution. Unfortunately, the development was plagued with problems, and to date only 35 of the contracted 50 devices have been delivered. These have proved to be somewhat unreliable because they are constructed with untested chips, which have a high infant mortality rate. On the basis of our experience, we have decided to abandon this form of the monitor, and we will not contract for additional hybrid monitors.

Since the 10 day monitor is essential to outpatient studies, the Section is now designing a new device based on a technology which uses packaged tested chips, and newly developed high density memories. It is anticipated that a device with 10 day memory and one year battery life can be produced in the size of the present 64 hour monitor. Initial production should begin late this year.

In the interim, the Section continues to produce the standard device, for new applications and to replace old monitors. More than 100 standard monitors are now in the field.

(2) Dyskinetic Mouth Movement Detector. A small, battery powered unit was developed for use in measuring, on a continuous basis, abnormal oral movements associated with tardive dyskinesia. Several accelerometer transducers were designed for the system to record the specific dyskinetic movement the patient might manifest, such as cheek puffing or lip pursing. The transducer output signal is connected to a modified Patient Activity Monitor which records the number of abnormal movements for the time interval selected, and store the number in memory to be read out later on a computer. A calibration box allows the investigator to determine the threshold point at which a movement is recorded. Two units were built; a one-minute interval unit which will store over 4 hours of data, and a second unit with a seven and a half minute interval which can store up to 32 hours of data.

(3) Parkinson's Disease Testing Station. Work has continued on developing instrumentation to be used in recording data on patients with Parkinson's disease. One of the neurological manifestations of the disease is tremor. New tri-axial accelerometers were developed that enable the tremor amplitude and frequency to be recorded and analyzed by the computer program. In order to verify the data recorded, a calibration system was developed including a mechanical device that will produce a 5 Hz sinusoidal movement at a fixed amplitude. This device can be used at the recording site to make sure the accelerometers and program are working properly. In addition, instrumentation was developed to provide more extensive calibration data in verifying acceleration constants at various amplitudes and frequencies.

The equipment used for the reaction and movement time measurements was also improved. New touch pads, circuitry and base were constructed to provide a more reliable and accurate means of recording these parameters.

(4) Exploratory Activity Cage. An activity cage has been developed to quantify the exploratory behavior of laboratory rodents. One-third of the area of a plastic animal cage forms a dark chamber which is separated from the lighted two-thirds by a partition. Four sets of IR diode emitters and phototransistor detectors positioned across an opening in the partition are used to count the number of times the animal crosses the partition and to measure the length of time spent in each compartment. Potential false transitions caused by tail movements in long-tailed rodents are rejected by the emitter-detector spacing geometry and the associated circuitry. It is hoped that this apparatus will provide a rapid, simple test for pharmacological studies of drug effects on exploratory activity.

(5) Rat Sleep Scorer. A device to categorize rat sleep states by real-time analysis of bifrontal and frontal-occipital electrocortical activity and electromyographic activity has been under development for several years. The algorithm which has evolved now achieves an acceptable scoring accuracy as verified by extensive tests. The scoring hardware has been interfaced to a line printer so that a formatted, chronological sleep-state record is produced to facilitate entry into a minicomputer for secondary analysis. Design of a multichannel system with complete, stand-alone analysis capability has been initiated.

(6) Electromagnetic Field Control System. The use of small sense coils within orthogonal electromagnetic fields has become a widely used method of recording horizontal and vertical eye movements. Vision studies involving long-term single unit recording place stringent requirements on the field-generating electronics. These include high frequency operation (20-30 KHz), both short and long-term stability in field strength and in field orthogonality, and the ability to maintain the stability of these parameters when moving metallic objects are within the fields. An electronic field coil system has been developed to operate at high frequency; it uses automatic gain and phase control to obtain the required operating stability. Independent adjustment of the two field strengths and the phase are provided.

(7) Rat Startle Response Measurement. Startle response, an important unconditioned reflex behavior, is used in studying how the nervous system is modulated by various environmental factors. A device was developed which accurately quantifies three parameters of the startle reflex: latency before response; time until peak force; and maximum peak force. The isometric force measurements are transduced by four semiconductor strain gages mounted in a double cantilever fashion. The device is both manually and microcomputer controllable, and will resolve forces from 10 to over 500 grams and times from 1 to 256 msec. Data can either be recorded manually from LED displays or by the microcomputer from a data bus for further analysis.

(8) Multichannel Programmable Stimulus Generator. This microprocessor-based instrument, currently under development, will provide a stimulus pulse output per lever press input during a period of time referred to as an epoch. The operator will be able to specify the duration of the epoch, the total number of epochs, the duration of the stimulus pulse in milliseconds, and the percent of time within a given epoch when stimulus pulses will be generated by a lever input. An additional feature will provide a listing of lever inputs per channel per epochs.

(9) Programmable Infusion Pump. This instrument, a motor driven syringe controlled by a microprocessor, can be programmed to provide pumping schedules which are various mathematical functions specified by the operator. When the appropriate input arguments are entered on a console, exponential, constant, and ramp infusion rates can be realized.

(10) Rat Activity Monitoring System: Solid-State Imaging System. The development of a new method of monitoring a rat's activity over a large area was begun. The activity cages using capacitance transducers, as described in previous annual reports, are only effective for areas up to 90mm square. In order to monitor rats in an enclosure of 1 meter square, a new technique was needed. It was felt that the activity could be monitored by a closed circuit TV system, but this was too expensive. A solid-state imaging system, consisting of a special lens system in conjunction with an 8x8 array of discrete phototransistors, was designed and is being tested. An overhead light casts a shadow of the animal on a translucent floor. This image is focused by the optical system on the phototransistor array in such a way that a single square of the floor, 1/8 meter on a side, projects onto a single phototransistor. Thus, it is possible to obtain an output of the transistor which is proportional to the amount of the square which is occluded.

COMPUTERS

The Instrumentation and Computers Section continues to support the use of the computer as a laboratory instrument. Small computers are used in the individual laboratories for on-line, real-time interaction, process control and data acquisition. ICS maintains support computers in Buildings 10 and 36. These systems provide means for program preparation, bulk storage, printing and plotting, and mathematical and statistical processing. Experimental data may be transmitted from the laboratory computers, via these systems, to the DCRT facilities for further processing. The support computers also serve to develop prototype systems and to test the feasibility of the use of a computer in specific laboratory applications. The latter capability allows an investigator, once he determines that the computer will do the job, to purchase an efficient system at minimal cost.

The Section provides software support for the individual investigators. A library of procedures has been developed that is tailored to the needs of the Intramural Program. Individual training is available for investigators with no prior experience in using or programming the computer. Computer specialists are available for consultation for all areas of computer use, programming, interfacing, real-time applications, time series analysis, data presentation, systems configuration and computer procurement. Although ICS does not provide an applications programming service, systems have been developed in collaboration with individual laboratories. Examples are included in the list of computer projects.

An increasing amount of time is devoted to program maintenance. Computer programs, especially in research related services, are not static and often require development, improvement or modifications. In addition to the software library and research related projects developed by ICS, much work is caused by the turnover of scientific and support personnel. Many systems developed by these persons prove useful to the laboratory. After they leave, maintenance of such

systems often falls to the personnel of ICS. The Section has investigated the use of structured programming techniques and programming languages in an attempt to define software standards. PASCAL was chosen as a standard language. As the problem of program maintenance continues to grow, use of these techniques will be necessary to enable the Section to furnish this service without an increase in personnel.

The workload on the ICS computer facilities has reached the saturation point. A second support computer for Bldg. 36 has been ordered. The Section has begun a system study for the possible acquisition of a new 32 bit minicomputer. Operating systems are available for these computers which support multiple users, but it is not clear that they may be implemented without adding personnel. They are particularly suitable for graphic image processing and digital signal processing. Techniques for digital signal processing are particularly applicable to neurophysiological data and such applications are continually under development by the Section. Even with the investigators working evenings and weekends, these applications take much time from program development and testing. The larger minicomputer system would provide greater precision, faster computation and multiple users. In addition, programs not requiring real-time data acquisition or process control could be developed on a system of this type.

One of the major functions of the Section is to provide systems studies for use of the computer as a laboratory instrument. The concept of the microcomputer as an integral part of laboratory instrumentation for process control, data logging, timing and coordination of instruments has proven to be both cost effective and efficient for laboratory applications. Mini- and microcomputer based systems provide a wide spectrum of tools for research ranging from lower cost data loggers to sophisticated systems which interact with the on-going experiment. These systems have resulted in a further integration of the engineering and computer functions of the Section and have enabled us to offer a wide range of computer related services. Projects such as the Parkinson's Disease Testing Station are the result of close coordination between the scientist, the electronic engineers, and the computer scientist and illustrates a type of service not feasible a few years ago.

IMAGE PROCESSING SYSTEM

The Instrumentation and Computers Section general purpose image processing system became operational in the spring of 1980. The system consists of a high-speed rotating drum scanner, an image array processor and display, and a PDP-11/60 computer. The drum scanner can digitize transparencies up to 10x10 inches with spatial resolution of 12.5 microns. The image array processor can simultaneously store, display, and manipulate up to three 512x512 digitized images. Images may be compared, superimposed, translated, zoomed or color coded at video rates. Images to be processed may be obtained by scanning autoradiographs, x-ray film, or photographic negatives, or by using previously digitized images generated by CAT or ECAT scanners.

An interactive, menu driven, software system is being developed to provide an extensive and expandable repertoire of basic image processing and input/output functions. Special purpose functions can be developed to meet specific user requirements. The facility is useful for numerous applications involving evaluation and quantification of biomedical images. Two applications, however, are primary: analysis of two-dimensional electrophoresis gels and analysis of autoradiographs of brain or tissue sections.

The autoradiographs are used for measurements of glucose utilization in brain tissue using the Sokoloff [^{14}C] deoxyglucose method of glucose substitution. Analysis of the autoradiographs involves displaying the digitized image on a TV monitor and outlining areas of interest. The average optical density is then computed and automatically converted into glucose utilization. Glucose utilization of brain regions as small as 100 microns in diameter can be computed. A color-coded glucose utilization map may also be produced.

Measurement of amino acid concentrations can be made using two-dimensional electrophoreses gels. The gels, which have been prepared by the appropriate stain and fixer, are photographed; or if radioisotopes are used, an autoradiograph is obtained. The film is scanned and digitized into an array of optical densities. The resultant image is then displayed and analyzed. Measurement of individual amino acid concentrations can be made by integrating optical density within a defined boundary. A test gel may be compared with a standard gel using the image array processor to determine the presence or absence of a particular substance.

Additional examples of computer projects include:

(1) Membrane Activity of Neurosecretory Cells. This system was developed in collaboration with the Laboratory of Neurophysiology (NINCDS) to study the conductance of cell membranes that often exhibit bursting activity during clamping. The system is being upgraded to record and monitor the tissue culture neurons on-line and to allow the iontophoretic or pressure injection of neuroactive substances onto the external surfaces of the cells. The purpose is to discover the nature of the action of such substances on the properties of the cell membrane.

(2) Cell Culture Analysis. This system is designed to provide an on-line analysis of tissue culture neurons. The first phase, to study the excitatory or inhibitory post-synaptic potentials of these cells, has been completed. A unique feature of this system is the on-line control of artifacts introduced by the measurement system and the properties of tissues in culture and to control the threshold levels and amplification level as the experiment is in progress. Visual displays of amplitude, integral and latency are available, as well as averaged evoked response. In addition, on-line monitoring of post-synaptic potentials elicited by stimuli presented in pairs or in trains of pulses are available. The system also studies spontaneously occurring miniature potentials. This system will be extended to allow analysis of the cells by other techniques such as voltage clamping and the iontophoretic injection of neuroactive substances on the surface of the cell.

(3) Rat Activity Monitoring System. The system designed to measure gross movements and rearing activity of white rats was extended to differentiate between two levels of gross movements and to monitor the amount of time the animal spends in each quadrant of the cage.

(4) Membrane Channel Analysis. Acetylcholine molecules outside neural membranes open and close channels allowing the passage of Na^+ and K^+ ions. Current fluctuations resulting from single channels opening and closing have a high signal-to-noise ratio. This program catalogues and identifies the duration and amplitude of channel activity, and provides a spectral distribution of the electrical activity of the membrane.

(5) Cytofluorographic Analysis of Single Cells. The system designed for cytofluorographic cell sorting by the Computer Systems Laboratory, DCRT, is being modified for the cytofluorographic analysis of cells in immunological studies.

(6) Process Control System. A process control system was developed to allow an aperture controlling a narrow beam of light to be projected across the visual field of the retina of experimental animals. The beam may be centered on the retinal cell from which a microelectrode is recording responses. The beam may be automatically passed across this light at controlled rates and controlled vectors. The system is designed to give the investigator flexibility in stimulus presentation, freeing him for observation of the response.

ENGINEERING, COMPUTER AND FABRICATION SERVICES

This table shows the distribution of the Section's workload among the various laboratories.

<u>LABORATORY OR BRANCH</u>	<u>HOURS</u>	<u>PERCENT</u>
Neurophysiology, NIMH - - - - -	3580	11.26
Clinical Psychobiology, NIMH - - - - -	3158	9.93
Clinical Science, NIMH - - - - -	2585	8.13
Biological Psychiatry, NIMH - - - - -	2280	7.17
Cerebral Metabolism, NIMH - - - - -	2092	6.58
Neuropharmacology, NIMH - - - - -	2052	6.45
Neurophysiology, NINCDS - - - - -	2025	6.37
Developmental Neurobiology, NICHD - - - - -	2015	6.34
Neuropathology and Neuroanatomical Sciences, NINCDS - - -	1986	6.25
Experimental Therapeutics, NINCDS - - - - -	1875	5.90
General and Comparative Biochemistry, NIMH - - - - -	1668	5.25
Biophysics, NINCDS - - - - -	1498	4.71
Neuropsychology, NIMH - - - - -	1437	4.52
Molecular Biology, NINCDS - - - - -	690	2.17
Brain Evolution and Behavior, NIMH - - - - -	650	2.04
Infectious Diseases, NINCDS - - - - -	596	1.87
Neurochemistry, NIMH - - - - -	507	1.59
Neurochemistry, NINCDS - - - - -	485	1.53
Neuro-Otolaryngology, NINCDS - - - - -	354	1.13
Neural Control, NINCDS - - - - -	259	.81
NIMH (Total)	20,009	62.94
NINCDS (Total)	9,768	30.72
NICHD (Total)*	2,015	6.34
	31,792	100.00

*NICHD loans the Section one position, and is thus entitled to 1700 hours of service.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Neuroepidemiology Section, ODIR
National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report
for Period October 1, 1979 through September 30, 1980
Neuroepidemiology Section
Office of the Director
Intramural Research Program
National Institute of Neurological and Communicative
Disorders and Stroke

Bruce S. Schoenberg, M.D., M.P.H., Chief

The Neuroepidemiology Section is responsible for the development and implementation of epidemiologic and genetic programs to investigate the cause, prevention, and treatment of neurologic disorders in human populations. Emphasis has been placed on major neurologic diseases in which the diagnoses can be clinically verified to the satisfaction of skilled neurologists.

The Section is unique in being the only unit devoted exclusively to research in the epidemiology of diseases of the nervous system. These research studies require collaboration of many individuals. However, since there is a severe shortage of available manpower in neuroepidemiology, the Section developed an active teaching program for current and future collaborative investigators. A series of four videotapes produced by the Section are distributed on a loan basis without charge. A textbook, entitled NEUROLOGICAL EPIDEMIOLOGY: PRINCIPLES AND CLINICAL APPLICATIONS, was published. A symposium on the solutions to methodologic problems in neuroepidemiology was held in conjunction with the Society for Epidemiologic Research and the World Federation of Neurology. Future courses are planned in collaboration with the American Academy of Neurology, the University of Minnesota, the World Health Organization, the University of Madrid, the University of Florence, the World Federation of Neurology, and the International Epidemiologic Association. We are also providing opportunities for fellows to spend six months to one year to work with members of the Section in order to learn the techniques of neuroepidemiology. During the past year we have had physicians from Great Britain, Nigeria, Mexico, and Turkey, and have received inquiries from China, Spain, and Israel for future opportunities. There is considerable neuroepidemiologic interest among senior neurologists (two of the physicians working in the Section are professors and chairmen of their own units abroad). Finally, current individual and institutional research training grant programs have recently been expanded to include neuroepidemiology. With the initiation of an educational program, the Section has focused on research investigations.

Epidemiologic studies have two basic requirements: uniformity and accuracy of data collection. This necessitates the use of a standardized, internationally accepted classification and coding system. The most recent scheme generated by the World Health Organization is seriously deficient with regard to neurologic disorders. The Section is therefore collaborating with the Charing Cross (London) Neuroepidemiology Unit, the World Health Organization Neurosciences Program, the World Federation of Neurology, and the American Academy of Neurology to revise this system of classification and improve its usefulness for neuroepidemiologic research.

Another important problem for the neuroepidemiologist is the enormous cost of maintaining neurologic surveillance on a large number of patients. Therefore, we have attempted to utilize existing registries of neurologic disease, such as in a study of presenile dementia based on the Israeli National Neurologic Disease Registry. In addition, we are in the process of organizing information routinely collected through the British National Health Service on all neurologic inpatients in a section of London with a population of 3-1/2 million inhabitants. The utility and accuracy of these data are being tested in ongoing studies of the Guillain-Barré syndrome and primary brain tumors.

There have been a number of neuroepidemiologic case-control studies which have suggested associations between a given factor and a particular disease, but the number of patients has been inadequate for meaningful conclusions. We are working in collaboration with a number of multiple sclerosis clinics to establish a uniform protocol and data base to enable us to explore several hypotheses of interest which require a large number of cases. Similar arrangements are being made to initiate analytic epidemiologic studies of Alzheimer's disease. These several projects in support of research activities, have been initiated in conjunction with a very active research program.

With regard to neurologic problems in children, the Section documented the frequency of primary intracranial neoplasms in the pediatric population of Rochester, Minnesota, and the State of Connecticut. In addition, we investigated cerebrovascular disease in infants and children. The magnitude of this problem was documented for the first time. The study demonstrated that neonatal intracranial hemorrhage is relatively common (1.1 cases/1,000 live births), that it is strongly associated with prematurity and hyaline membrane disease, and that it is difficult to recognize clinically. For pediatric cerebrovascular disease unassociated with birth, trauma, or infection, the incidence rate was 2.5/100,000/year. These cases were further characterized by survival, residual

disability, and cause (whenever possible). The clinical and angiographic features of children with moyamoya disease were examined in detail. This condition appears to be more common than suggested by early case reports. The Section is also studying cerebral palsy in a defined population, to determine if new developments in perinatal care have resulted in changes in the incidence or clinical pattern of these disorders.

The Section has conducted extensive investigations of primary intracranial neoplasms. First, problems with nomenclature and disease definition were resolved. After this, two patterns of age-specific incidence emerged. Analyses of most population-based data worldwide revealed a small childhood peak, followed by a later peak between ages 50 and 80. Data for Rochester, Minnesota, however, showed the childhood peak, followed by an increasing incidence rate with increasing age. Careful study of this discrepancy showed 1) that the greater percentage of cases first diagnosed at autopsy in Rochester accounted in large part for this difference, and 2) that a substantial number of brain tumors remain undiagnosed in the elderly during life. Studies have just been completed to evaluate the role of computerized tomography in the diagnosis of brain tumors and to explain the recent increase in the incidence of pituitary tumors among women of child-bearing age. The introduction of computerized tomography has not resulted in any increase in the reported frequency of these tumors in the Rochester, Minnesota population, while the apparent rise in the incidence of pituitary tumors seems to be the result of more sophisticated neuroendocrine diagnostic procedures. An exhaustive, critical review of a survey strategy to measure the national incidence and prevalence of intracranial neoplasms has been completed. In addition, racial differentials in the frequency of certain intracranial tumors (meningiomas and pituitary adenomas) are being examined. Investigations of the relationship between intracranial neoplasms and extracranial tumors have been especially rewarding. An association was found between the occurrence of breast cancer and meningioma in women. This result raises interesting etiologic possibilities when considered with other evidence: 1) meningioma is the only common intracranial neoplasm with a higher incidence in females; 2) the abrupt clinical appearance or enlargement of this tumor during pregnancy has been described; and 3) the finding of estrogen receptor protein in meningioma has been reported.

At the present time, there is little to suggest that improved medical management of the completed stroke will substantially affect the cerebrovascular disease problem. It would appear that greater benefit could be achieved by dealing with the precursors of stroke rather than delaying treatment

until after the event has occurred. Therefore, a non-concurrent, prospective study of a cohort of 2,000 elderly individuals was undertaken to determine the role of heart disease and hypertension as risk factors for both transient ischemic attacks and completed stroke. Data editing has been completed and analysis of these data have just been finalized. Transient ischemic attacks and completed ischemic strokes are revealing different patterns of risk factors. In addition, certain unusual patterns of cerebrovascular disease (e.g., more than 20 TIA's/day) are being studied.

Alzheimer's disease/senile dementia, despite its high apparent clinical frequency among the elderly, has not been well studied in a U.S. population. Because of this, we have launched three investigations. One is derived from a review of detailed clinical records utilizing a population-based, records-linkage system; the second utilizes a two-stage survey consisting of a questionnaire and clinical examination; and the third (in collaboration with the National Institute on Aging) is based on a questionnaire survey. Data collection on the first two investigations have been completed and are currently being analyzed. These investigations should provide better estimates of the magnitude of the disease and its distribution in the population. These will likely yield etiologic hypotheses which can be further studied using the case-control approach, and will provide the opportunity to investigate dementia occurring with other neurologic disorders such as Parkinson's disease.

There has been some debate as to whether Alzheimer's disease is a single disease entity regardless of its age at presentation. Since the frequency of Alzheimer's disease is relatively low before age 60, an enormous population is required for surveillance in order to obtain an adequate number of patients for study. We are therefore utilizing the resources available through the Israeli National Neurologic Disease Registry to identify all potential cases among the population of Israel. These cases will be intensively reviewed to determine the accuracy of diagnosis and to explore a number of epidemiologic studies of the distribution and risk factors for this disease. A similar sex-ratio for patients with onset before and after age 60, and a steadily increasing age-specific incidence in the elderly would argue in favor of a single disease entity.

The Section is also interested in accurately documenting possible racial differentials in the prevalence of major neurologic disorders. A number of early investigations suggested possible differences by race, but were based on hospital or clinic experience and could not identify a well-defined population from which cases were derived.

Population-based studies followed, but questions concerning the results centered on possible racial differentials in access to expertise in neurologic diagnosis and treatment. We reinvestigated (in conjunction with the Surveys and Demographic Studies Section) this problem of possible racial differentials in the prevalence of major neurologic disorders by surveying a well-defined population (approximately 25,000, almost equally divided between blacks and whites). We developed a strategy which eliminated the requirement that persons must have entered the health-care system for detection of disease. The disorders investigated included cerebral palsy, dementia, psychomotor delay, epilepsy, Parkinson's disease, and cerebrovascular disease (both transient ischemic attacks and completed stroke). The basis of the investigation was a door-to-door survey which utilized a detailed questionnaire inquiring not only about diagnoses, but also about signs and symptoms suggestive of neurologic dysfunction. Over 99% of the households agreed to the interview. Those household members suspected of having one of the disorders of interest were then asked to have a neurologic examination conducted by a senior, board-certified neurologist. The interviews and examinations have been completed, and the data are being edited and analyzed. Similar strategies are being developed for application in developing countries (e.g., Nigeria, Mexico, Turkey), in collaboration with the World Health Organization.

We currently have very little information on the patterns of medical care received by all individuals with neurologic disease in a given community. The Section is, therefore, studying this problem in Rochester, Minnesota. Although the findings of this investigation will not necessarily be applicable to other regions of the U.S., the City of Rochester does offer particular advantages. Cases of neurologic disease among residents have already been identified through previous studies. Medical encounters are easily documented through a records-linkage resource. In addition, Rochester residents have access to high-quality medical care, and physicians with neurologic expertise are available within the community. Thus, the Rochester experience may provide some estimate of the pattern of medical care in the ideal situation in which the population has ready access to neurologic expertise, and in which there is little financial restraint to such care. The study for patients with brain tumor is being prepared for publication, and similar data are being analyzed for completed stroke.

Although death certificate data are limited by possible misdiagnosis, incomplete case ascertainment, errors in coding, etc., detailed morbidity information on neurologic diseases for the entire U.S. and for other countries is not

available. The Section has analyzed mortality data for selected neurologic disorders by country and by county in the U.S. The overall patterns which emerge may be useful in evaluating trends over time and in formulating etiologic hypotheses. Among the most interesting findings is that the mortality from cerebrovascular disease has decreased in most developed countries over a 20-year period. This trend is not universal, however. For multiple sclerosis, countries initially reporting high mortality rates have generally reported declines while those with low rates earlier are reporting increases, so that more recent mortality data for multiple sclerosis by country show less of a differential than previously reported.

A number of other collaborative projects include the investigation of space/time clusters of neurologic disease (with the Center for Disease Control), the development of survey strategies (with the World Health Organization and the Section on Disease Statistics Surveys), a study of myasthenia gravis and multiple sclerosis in the same patient (with the Mayo Clinic), an investigation of neurologic disorders during pregnancy and the postpartum period (with the Mayo Clinic), a study of the epidemiology of eye tumors (with the Connecticut State Department of Health), the effect of weather on the incidence of stroke (with the Mayo Clinic), and international comparisons in the incidence of brain tumors. Finally, extensive reviews have been prepared on the epidemiologic aspects of Huntington's disease, otitis media, Alzheimer's disease, and cerebrovascular disease.

The clinical neurogenetics component of the program involves three areas: 1) genetic-epidemiologic studies of multifactorial neurologic disorders (e.g., Parkinson's disease, multiple sclerosis (MS), and Alzheimer's disease); 2) genetic and biochemical studies of hereditary nervous system tumors; and 3) genetic studies of the dystonias, Tourette's syndrome, and other movement disorders.

Contributions were made to the understanding of Parkinson's disease and multiple sclerosis. We have completed a preliminary study of 15 monozygotic twin pairs, selected on the basis of at least one member of the twin pair being diagnosed as having Parkinson's disease. Surprising was the observation that in 12 monozygotic twin pairs, there was no concordance for Parkinson's disease. Although the unaffected co-twin in each case remains at risk for Parkinson's disease, these preliminary observations suggest that environmental factors rather than genetic factors are more critical. Data also suggested that smoking is associated with a decreased risk for parkinsonism and that in

most instances long-term personality differences exist between the affected and the unaffected twin (i.e., the Parkinson twin being the more inhibited).

In multiple sclerosis we reported on 30 MS twin pairs. Concordance for multiple sclerosis was 66 percent among monozygotic twins 50 years of age or older and 40 percent for all monozygotic twin pairs. The corresponding figures were 0 percent concordance for dizygotic twins over age 50 and 15 percent for dizygotic twins of all ages. These findings suggest that there is a significant genetic contribution to multiple sclerosis. However, the number of older twin pairs must be expanded. Haplotypes HLA-A₃ and B₇, as well as the antigen DW₂, were increased in this population. However, a pair of monozygotic twins, age 56, discordant clinically for MS were both DW₂ positive and HLA-B₇ positive, indicating that the presence of these genes or an "MS gene" in this area of chromosome 6 is not sufficient for development of the disease.

Two developments in the area of hereditary tumors of the nervous system are of note. We have recently documented that there is a central form of neurofibromatosis with bilateral acoustic neuroma, distinct from the disease originally described by von Recklinghausen in which peripheral manifestations predominate. Collaborators have now evaluated nerve growth factor in serum from members of three large kindreds with the central form of the disease. The data from this and related studies suggest that basic to both forms of neurofibromatosis are alterations in the active subunit of nerve growth factor. Difficulties in nerve growth factor assay require that further study by a well-established laboratory be carried out to confirm this finding. In 1979, we organized a multidisciplinary project utilizing the resources of the Interinstitutes Genetics Clinic, NIH. Specialists representing over 10 disciplines are participating in a comprehensive evaluation of "neurofibromatosis families" from the Washington, D.C. area. Coverage of this clinic by local newspapers and television resulted in ascertainment of over 100 new cases in a 5-day period.

In our study of movement disorders, two observations are of special note. Hydroxylase cofactor (tetrahydrobiopterin), which plays a critical role in the synthesis of the neurotransmitters dopamine, noradrenalin, and serotonin, was found to be markedly reduced in the cerebrospinal fluid of four members in one family affected with dystonia. Since there have been no consistent structural abnormalities in the brains of such patients, this finding may reflect a chemical alteration fundamental to dystonia. In Tourette's syndrome a comprehensive evaluation of the first 50 patients seen in the

"Tourette Clinic" run by NIMH, is complete. The following results were noted: a) in one-third, multiple family members were affected; b) in the majority there was inappropriate social behavior; and c) a positive response to Haloperidol was correlated with positive family history. The first two findings support original work by us, which at the time was at variance with the prevailing view of this syndrome. The third observation suggests that those with positive family history reflect a distinct biochemical entity.

CONTRACT NARRATIVE
Neuroepidemiology Section
Office of the Director, Intramural Research Program, NINCDS
Fiscal Year 1980

MASSACHUSETTS GENERAL HOSPITAL (N01-NS-4-2321)

Title: Multiple Sclerosis in the Shetland and Orkney Islands
and Caithness, Scotland

Contractor's Project Director: Dr. Raymond D. Adams
Professor, Dept. of Neurology

Contractor's Co-director: Dr. David C. Poskanzer
Associate Professor
Dept. of Neurology

Current Annual Level: None - Contract expired in October
1977.

Objective: An epidemiologic, virologic, and immunologic study of multiple sclerosis was undertaken in the Orkney and Shetland Islands, Scotland, where the rates of the disease are 309 per 100,000 and 184 per 100,000, respectively, as compared with the estimated prevalence rate of 40 per 100,000 in Boston. A search for exogenous etiologic factors and factors which might implicate heredity was undertaken.

All patients with multiple sclerosis in the Shetland and Orkney Islands have been identified, and two sets of appropriate controls were selected for each patient. For such individuals (both patients and controls), as well as certain family members of these individuals, previous history of infection (confirmed by serology), dietary history, sanitation history, history of exposure to animals, occupational history, travel history, and history of allergic diatheses were obtained, and family pedigrees were traced to 1776. Blood samples were obtained from patients, their two age- and sex-matched controls, and family members for (1) the histocompatibility determinants HLA, MLC, and B-cell alloantigen Ag7a; (2) blood group typing, red cell enzymes and serum proteins; and (3) viral antibody titers (rubeola, rubella, mumps, varicella, cytomegalovirus, herpes 1 and 2, Coxsackie B3 and B4, parainfluenza 1, 2, and 3, poliomyelitis 1, 2, and 3, echo 4 and 9, and EB virus). The blood samples were shipped to appropriate laboratories for study.

The establishment of family pedigrees and determination of blood groups, as well as their analysis, was sub-contracted (see contract N01-NS-6-2337). The principal investigators of contract N01-NS-4-2321 and contract N01-NS-6-2337 are cooperating in the interpretation and publication of results.

Major Findings: Findings determined to date were reported during the American Academy of Neurology meetings in April 1977 and 1979. The findings reported in the 1977 meeting were as follows:

(1) A bimodal age at onset curve was observed for Orkney Islands with two distinct peaks at ages 20 to 25 and 35 to 40. From this observation it became apparent that there is a subgroup of patients who share the following characteristics: same sex, early age at onset, temporal course of exacerbating-remitting disease, and the occurrence of HLA-B7. This group of patients, referred to as Multiple Sclerosis Type I, can be quite clearly distinguished from other forms of multiple sclerosis and may account for the disparities and variations in tissue typing present when the disease is perceived as if it were homogeneous, rather than at least two clinical, epidemiologic, laboratory, and possibly etiologic entities.

(2) The absence of optic neuritis as an isolated entity without subsequent evidence of other lesions of the nervous system in these islands is remarkable.

(3) Female Orkney patients who are HLA-B7 positive appear to have a lower titer response to several of the viruses studied, especially measles, as compared to female controls with HLA-B7 and other Orkney patients without this specificity.

(4) No virus of those studied is clearly associated with multiple sclerosis, either by antibody titer or the presence or absence of previous exposure by history to each virus.

The findings reported in the 1979 American Academy of Neurology meeting are as follows:

(1) Genetic analysis in collaboration with Roberts demonstrated a "heritability" of MS of 31 percent.

(2) HLA analysis in collaboration with Terasaki did not show a predominant type or trend.

(3) Studies for a long series of common viruses in collaboration with Sever did not demonstrate the putative agent.

(4) Demographic analyses in collaboration with Taylor and Illsley indicated that the high prevalence in the Islands was not the result of patterns of population movement.

The Orkney and Shetland Islands were shown to have the highest rates of MS recorded, though increases in the prevalence over time were largely a function of increasing

life expectancy of patients. Availability of controls born in the same year and in the same parish as the patient, and lifetime information about residence and travel of all subjects provided clear evidence of temporal-spatial clustering of MS patients in Orkney at two points in time: (1) about 21 years prior to onset, and (2) at the time of onset. The data suggest that not one, but two, environmental factors play a role in the etiology of MS. They may represent two discrete exposures or one exposure occurring twice in the lifetime of patients.

Significance to the NINCDS Program and Biomedical Research:

To date research on the etiology of multiple sclerosis follows a divergent path; the quests in search of environmental and genetic factors continue at comparable rates. It was anticipated that a study of genetic and environmental factors in a defined population with the world's highest reported prevalence of multiple sclerosis would provide guidelines for future research, justifying emphasis on the pursuit of either genetic or environmental factors.

Proposed Course of the Project: The contract expired on October 15, 1977. According to contract specification, the five manuscripts resulting from this study were submitted to the Institute. Recently, one of these manuscripts was published jointly by the principal investigators of contracts N01-NS-4-2321 and N01-NS-6-2337. The reference and summary of this paper is cited in the N01-NS-6-2337 contract narrative. Publication plans for the other four manuscripts remain uncertain.

CONTRACT NARRATIVE
Neuroepidemiology Section
Office of the Director, Intramural Research Program, NINCDS
Fiscal Year 1980

THE UNIVERSITY OF NEWCASTLE UPON TYNE (N01-NS-6-2337)

Title: Genetic Study of Multiple Sclerosis in the Orkney and Shetland Islands

Contractor's Project Director: Dr. D.F. Roberts, Professor
Dept. of Human Genetics

Current Annual Level: None from the 1979 budget. A sum of \$39,179 was allotted to this contract out of the 1976 budget. This sum was disbursed to Dr. Roberts.

Objective: (See contract N01-NS-4-2321 for an introductory statement)

Family pedigrees of multiple sclerosis patients and their controls were established for Shetland and Orkney. Blood groups, red cell enzymes, and serum proteins on specimens of blood obtained during the March 1976 field trip to the Orkney Islands were determined. The pedigree as well as all serology data for Orkney and Shetland were analyzed in a study of the genetic aspects of multiple sclerosis among the inhabitants of the Orkney and Shetland Islands.

Major Findings: Three papers were recently published:

- 1) Roberts, D.F., Roberts, M.J., and Poskanzer, D.C.: Genetic analysis of multiple sclerosis in Orkney. J. Epidemiol. Community Health 33: 229-235, 1979.

(In a family study of all patients with multiple sclerosis (MS) in Orkney, the number of inbred among patients, although high for Britain, is not higher than the number among controls, and the inbreeding coefficients appear to eliminate recessive involvement of rare genes from the aetiology. The kinship coefficients show that the ancestries of patients and controls are closely enmeshed, and eliminate from the aetiology involvement of recently introduced genes dominant or codominant in effect. Family histories show that single locus inheritance is unlikely unless penetrance is very low. Multifactorial genetic involvement is much more likely, and it is compatible with all recent findings; on this hypothesis heritability estimates, not altogether satisfactory because

of the limited number of patients in the population, suggest that the genetic contribution to the aetiology of the disease in Orkney is only moderate.)

- 2) Roberts, D.F., Papiha, S.S., and Poskanzer, D.C.: Polymorphism and multiple sclerosis in Orkney. J. Epidemiol. Community Health 33: 236-242, 1979.

(Study of the blood group, isoenzyme, and serum protein systems representing polymorphic variants at 23 loci, in a population of 53 multiple sclerosis patients in Orkney, their relatives, and a control series, showed that patients were neither more homozygous nor more inbred than controls. Any possible association of the disorder with the ABO and rhesus blood groups was not directly causal, but was related to the families of the patients rather than to the patients themselves.)

- 3) Al-Agidi, S.K., and Roberts, D.F.: Serum immunoglobulin levels in multiple sclerosis in Orkney. Acta Neurol. Scand. 60: 320-327, 1979.

(Serum levels of immunoglobulins A, G and M in the population of multiple sclerosis patients in Orkney were generally similar to those in series of contiguous and discontiguous controls, and in the normal first-degree relatives both of patients and controls. There is a slight elevation of mean log IgE in patients, and this is due mainly to elevation in the rural patients. Factors possibly responsible are sought, but none can be clearly identified.)

In summary, the findings of these three studies do not support heredity as an etiologic factor.

Significance to the NINCDS Program and Biomedical Research:
(See this item as stated for contract N01-NS-4-2321.)

Proposed Course of the Project: The contract expired on June 26, 1977, and all reports required by the contract were received by the Institute.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01924-10-ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Genetic Studies of the Torsion Dystonias and Other Disorders of Movement</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Roswell Eldridge Other: Thelma Koerber Seymour Kaufman Linda Nee Ronald Polinsky	Geneticist Statistical Assistant Chief Social Worker Staff Associate	NES ODIR NINCDS NES ODIR NINCDS LNC IRP NIMH LCS IRP NIMH LCS IRP NIMH
COOPERATING UNITS (if any) Laboratory of Clinical Science, NIMH Laboratory of Neurochemistry, NIMH		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Clinical Neurogenetics Studies, Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <p style="text-align: center;">0.75</p>	PROFESSIONAL: <p style="text-align: center;">0.25</p>	OTHER: <p style="text-align: center;">0.5</p>
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SUMMARY OF WORK (200 words or less - underline keywords) <p> In this project we seek to clarify and expand the nosology of the hereditary movement disorders, contribute to the understanding of their underlying biochemical basis, determine the most effective treatment for each and suggest guidelines for counseling individuals at risk. General syndromes under study include the dystonias, tic disorders including Tourette syndrome, Huntington's chorea and myoclonus. Approaches include standard epidemiologic and clinical genetic studies together with collaborative efforts in evaluating the role of neurotransmitters such as dopamine, their precursors, and metabolites, and their necessary cofactors. </p> <p>Former title: (Genetic Studies of the Torsion Dystonias, Tourette Syndrome and Other Disorders of Movement).</p>		

Project Description:

Objectives: Included among the disorders of movement such as the choreas, the dystonias, and tic syndromes are a number of discrete diseases which are due to a single gene mutation. Examples of mutations producing autosomal dominant traits are Huntington's chorea and one form of torsion dystonia. Examples of mutations leading to autosomal recessive traits are Lafora type myoclonic epilepsy and the type of torsion dystonia responsible for most cases in the Jewish population.

In this project we seek to uncover additional specific diseases within general movement disorder syndromes, contribute to the understanding of their underlying biochemical basis, determine the most effective treatment for each and suggest guidelines for counseling individual family members.

Methods Employed: Initially, families with members exhibiting a particular syndrome undergo detailed clinical evaluation. Extensive genealogic data is then analyzed in conjunction with clinical observations and relevant laboratory studies. A nosologic classification is prepared. Promising biochemical leads are explored in collaboration with established investigators. Simultaneously, existing treatment programs are evaluated, and where indicated, there are therapeutic trials of new agents.

Major Findings: During the past year, a comprehensive review has been published concerning Gilles de la Tourette syndrome. The review was based on a study of 50 selected families evaluated at the Clinical Center, NIH, with Tourette syndrome. These studies indicated: a familial concentration of cases, particularly in those responsive to Haloperidol; history of frequent transient motor and vocal tics in female relatives of male Tourette patients; and inappropriate social behavior in many.

Low hydroxylase cofactor was found in spinal fluid in 6 members of one "dystonia family."

Significance to Biochemical Research and the Program of the Institute: Individually these disorders are uncommon but collectively the hereditary disorders of movement represent one of the major public health problems. In addition, information gained from analysis of these discrete genetic traits may contribute understanding to the cause and treatment of more common problems, such as Parkinsonism, in which the genetic constitution may be only one of several contributing factors.

Proposed Course: Continue search for distinct entities within movement disorders syndromes seeking their biochemical basis, specific therapy and prevention. A screening test for cofactor alteration in urine in various forms of dystonia awaits addition of appropriate staff.

Publications:

Nee, L.E., Caine, E.D., Polinsky, R.J., Eldridge, R., and Ebert, M.H.: Gilles de la Tourette Syndrome. Clinical and Family Study of 50 Cases. Ann. Neurol. 7: 41-49, 1980.

Williams, A., Eldridge, R., Levine, R., Lovenberg, W., and Paulson, G.: Low CSF Hydroxylase Cofactor Levels in Inherited Dystonia. Lancet II: 410, 1979.

Honors and Awards:

Invitation to serve as Medical Advisor, Pennsylvania Chapter, Dystonia Research Foundation of North America.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01927-10 ODIR																														
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SUMMARY OF WORK (200 words or less - underline keywords) <p>In this project we seek: to define and classify <u>hereditary tumors</u> of the <u>nervous system</u> in addition to the 9 such diseases already recognized; to add to the <u>clinical</u> description and <u>natural history</u> of these diseases; to suggest methods for early diagnosis; <u>evaluate present modes of treatment</u>; and develop methods for <u>preclinical detection</u> and <u>screening</u>.</p>																																

Project Description

Objectives There are at least nine genetically determined syndromes which include as one of their chief manifestations tumors of the nervous system. Peripheral neurofibromatosis and tuberous sclerosis are among the more common examples. It is the objective of this project to document additional hereditary traits which can cause such neoplasms; add to the clinical description and natural history of such traits; suggest effective means of early diagnosis; evaluate various modes of treatment and develop methods of preclinical detection and screening.

Methods Employed: In families with two or more individuals affected with the same rare tumor of the nervous system, members undergo clinical, genealogic and radiologic evaluation. Appropriate physiologic and biochemical studies are carried out in collaboration with laboratory investigators.

Major Findings: We have documented the existence of "Central Neurofibromatosis," the hallmark of which is the presence of Bilateral Acoustic Neuroma. Recently, we have reported on clinical and genetic findings in over 130 individuals with this trait.

In collaboration with the Department of Medicine, Johns Hopkins Hospital; Laboratory of Viral Carcinogenesis, NCI; and Department of Neurology, Mount Sinai College of Medicine, we are evaluating the usefulness of nerve growth factor in serum as a means of preclinical detection. To date, nerve growth factor has been evaluated in 30 affected individuals and their relatives from three kindreds previously studied by us. A major need of nerve growth factor assay is to improve its reproducibility, even at low concentrations.

Significance to Biochemical Research and the Program of the Institute: Hereditary tumors of the central nervous system are generally treatable if diagnosed early. Radiologic and physiologic techniques permitting early diagnosis would be of great use. Since many of these hereditary tumors are autosomal dominant in their inheritance pattern with onset during or after the childbearing years, there are individuals in such kindreds who carry a 50 percent risk of developing the trait who are faced with the question of family planning. Such individuals would gain immediate benefit if reliable, noninvasive, predictive tests were developed. Also, knowledge gained in the course of this practical application should contribute to understanding the mechanisms of tumor development.

Proposed Course: A report is in preparation summarizing our clinical and genetic findings in 130 affected individuals with central neurofibromatosis. The utility of nerve growth factor as a preclinical detector in individuals at risk should be clarified.

Comprehensive screening programs for neurofibromatosis, von Hippel-Lindau syndrome and other hereditary nervous system tumors will be undertaken.

Publications:

Fabricant, R.N., Todaro, G. J., and Eldridge, R.: Increased Levels of a Nerve-Growth Factor Cross-Reacting Protein in "Central" Neurofibromatosis. Lancet I: 4-7, 1979.

Eldridge, R.: Neurofibromatosis (von Recklinghausen disease): Genetics, Cell Biology, and Biochemistry. In Riccardi, V.M., and Mulvihill, J.J. (Eds.): Advances in Neurology. New York, Raven Press, in press.

Honors and Awards

Invitation to R. Eldridge to present lecture: "Central Neurofibromatosis and Nerve Growth Factor." American Society of Human Genetics, Sept. 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02167-06 ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Genetic Epidemiology Studies in MS and Other Multifactorial Neurologic Disorders</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Roswell Eldridge Other: Thelma Koerber Susan Ince Dale McFarlin Henry McFarland Christopher Ward Donald Calne James Dambrosia	Geneticist Statistical Assistant Geneticist Chief Assistant Chief Visiting Associate Chief Mathematical Statistician	NES ODIR NINCDS NES ODIR NINCDS NES ODIR NINCDS NI IRP NINCDS NI IRP NINCDS ET IRP NINCDS ET IRP NINCDS OBFS OD NINCDS
COOPERATING UNITS (if any) Department of Neurology, University of Mississippi Department of Neurology, Rutgers University, Piscataway, N.J. ET, NI, IRP and OBFS, OD, NINCDS		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Clinical Neurogenetics Studies, Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <p style="text-align: center;">2.5</p>	PROFESSIONAL: <p style="text-align: center;">0.5</p>	OTHER: <p style="text-align: center;">2</p>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p>In this project we are coupling genetic study of <u>selected families and twin pairs</u> with epidemiologic, immunologic, serologic and neurochemical studies of <u>disorders due to multiple factors</u> such as <u>multiple sclerosis</u> and <u>Parkinsonism</u>. This approach should <u>clarify the etiology</u> of these diseases, indicate <u>individuals or populations at high risk</u> and suggest a mechanism for <u>prevention and treatment</u>.</p> <p>To date, over 20 presumptive "<u>Multiple Sclerosis</u>" families and over 100 <u>twin pairs</u> with this condition have been ascertained. <u>Over 115 twin pairs</u> with Parkinsonism have been ascertained.</p> <p>Former title: (Infectious, Immunogenetic, Epidemiologic and Clinical Studies in Multiple Sclerosis, Parkinsonism and Other Multifactorial Neurologic Disorders).</p>		

Project Description

Objectives: As the genetic control of immune response becomes clarified, new avenues of exploring diseases such as multiple sclerosis (MS) are suggested. Improved understanding of the chemical and cellular changes underlying Parkinsonism also permits new approaches to its study. Our objective is to couple this new understanding with genetic and epidemiologic techniques in order to clarify disease mechanism, indicate high-risk individuals and populations and suggest possible means for prevention and treatment.

Methods Employed: Formal techniques of clinical genetics, neurochemistry, serology, and immunology will be merged. Selected populations including families with multiple members or twin pairs affected with the disease will be studied in depth. Unaffected family members, unaffected twins, and spouses will serve as controls. Specific investigations may include: detailed history and neurologic examination, computerized axial tomography; dermatoglyphic analysis; genotyping of blood for red cell antigens, serum proteins and the A, B, and D loci of the major histocompatibility complex; serum studies of viral antibody, immunoglobulin levels and complement levels; spinal fluid examination for routine elements plus determination of immunoglobulin content, oligoclonal banding, presence of myelin basic protein; and cellular study of migration inhibition and mixed lymphocyte culture response and genotyping of "B" lymphocyte.

Major Findings: Most impressive has been the difficulty in ascertaining bonafide MS families, and twins with either MS or Parkinsonism. Given the frequency of twinning and the frequency of MS in Parkinsonism, over 1,000 twin pairs with each disorder would be predicted in the United States. Utilizing a variety of ascertainment techniques including patient and physician contact, notices in medical and lay publications, and base-twin registries, less than one-fifth of the predicted number of twin pairs have been found for each condition.

Our detailed study of 14 MS families and 30 MS twin pairs has resulted in the following preliminary conclusions. In half of the families and several of the twins, diagnosis of MS could not be confirmed clinically. Thus, careful clinical documentation is an essential prerequisite in any patient-based study of this disorder. No consistent segregation of HLA type was noted between affected and unaffected family members. Thus, there is not a single, major gene with the HLA complex whose presence is sufficient and necessary for the development of MS.

Of 24 MS twin pairs, 6 were MZ concordant, 6 were MZ discordant, 2 were DZ concordant, and 10 were DZ discordant. This increased concordance rate in MZ twins suggests genetic factors are important - but in association with certain environmental events. The one monozygotic twin pair over 50 years of age, which was discordant for MS, was concordant for the HLA-DW₂ antigen which is associated with MS in an unusual frequency. Most of those unaffected in the dizygotic twin pairs and 2 of 6 unaffected monozygotic twin pairs had one or more oligoclonal bands, suggesting a subclinical nervous system involvement.

A preliminary report based on 12 monozygotic twin pairs discordant for Parkinsonism has been presented. Affected twins, in general, smoked less and had more introverted personalities than their unaffected cotwins.

Significance to Biomedical Research and the Program of the Institute: Disorders in which both genetic and environmental factors contribute, such as MS and Parkinsonism, comprise a major neurologic public health problem. Ample evidence from data based on populations already indicates genetic factors have a role in their causation. By coupling existing knowledge of genetics, the immune response, and neurochemistry, understanding of this group of disorders should be advanced, methods for prevention and treatment suggested and the risk for these diseases in close relatives assigned more accurately.

Proposed Course: Ascertainment of MS families, MS twin pairs, and Parkinson twin pairs continues. The first phase of the MS family and twin studies is nearing completion. A presentation of the clinical and laboratory observations based on 30 MS twin pairs is in preparation. Genetic and epidemiologic reports based on 56 twin pairs will follow.

The second phase of these studies will focus on appropriate epidemiology and laboratory studies in selected genetic groups in which the MS or Parkinson phenotype can be assigned definitely.

Ascertainment of twins with Alzheimer's disease is underway.

Publications

Williams, A., McFarland, H., Eldridge, R., Houff, S., Krebs, H., and McFarlin, D.: Clinical and Immunologic Studies on Selected Twins With Multiple Sclerosis. Neurology 29: 573-574, 1979.

Duvoisin, R.C., Eldridge, R., and Williams, A.: A Twin Study of Parkinson's Disease. Neurology 29: 578-579, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02240-04 ODIR
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Epidemiology of Dementia		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Bruce S. Schoenberg Chief NES ODIR NINCDS		
COOPERATING UNITS (if any) Epidemiology, Demography, and Biometry, NIA; W. Massey, M.D., Duke University; E. Kokman, M.D. and J.P. Whisnant, M.D., Mayo Clinic; B. Jordan, Harvard Medical School; M. Alter, Temple Univ.; E. Kahana, Hadassah Hospital, Jerusalem, Israel		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) A number of different approaches are being utilized to estimate the <u>mortality</u> and <u>morbidity</u> of <u>Alzheimer's disease/senile dementia</u> in several population groups in the U.S. and to measure the distribution of this disease in segments of the population.		

Project Description:

Objectives: To obtain estimates of the magnitude and distribution of Alzheimer's disease/senile dementia in segments of the U.S. population.

Methods Employed: Mortality information is obtained from death certificate data for the U.S. Four morbidity studies are also currently underway. One is derived from a review of detailed clinical records utilizing a population-based, records-linkage system; the second utilizes a two-stage survey consisting of a questionnaire and clinical examination; the third (in collaboration with the National Institute on Aging) is based on a questionnaire survey; and the fourth uses records available from a population-based registry serving an entire county.

Major Findings: The mortality data reveal an increasing death rate with increasing age, but probably represent underascertainment to a great extent. Data from the first two of the morbidity studies have been collected and are undergoing analysis. All other studies are still in the data-collection phase.

Significance: Alzheimer's disease/senile dementia, despite its high apparent clinical frequency among the elderly, has not been well-studied in a U.S. population. These descriptive studies will likely yield etiologic hypotheses which can be further investigated using the case-control approach, and will provide the opportunity to investigate dementia occurring with other neurologic disorders, such as Parkinson's disease. These studies should also provide evidence for whether Alzheimer's disease occurring in the very elderly and that occurring in the presenium represent the same disease process.

Proposed Course of Project: Data collection will continue during the coming year. Two of the studies are at a stage in which data analysis may be expected to begin during the coming fiscal year.

Publications:

Schoenberg, B.S.: Methodologic approaches to the epidemiologic study of dementia. In: Schuman, L.M., and Mortimer, J.A. (Eds.): Epidemiology of Dementia. London, Oxford University Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02241-04 ODIR															
PERIOD COVERED October 1, 1979 through September 30, 1980																	
TITLE OF PROJECT (80 characters or less) The Epidemiology of Cerebrovascular Disease in Adults																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: Karin A. Rosenblatt</td> <td>Epidemiologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> <tr> <td>Rudy Capildeo</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: Karin A. Rosenblatt	Epidemiologist	NES	ODIR	NINCDS	Rudy Capildeo	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS													
Other: Karin A. Rosenblatt	Epidemiologist	NES	ODIR	NINCDS													
Rudy Capildeo	Neurologist	NES	ODIR	NINCDS													
COOPERATING UNITS (if any) J.P. Whisnant, M.D., Mayo Clinic; D.G. Schoenberg, M.S., Bethesda, Maryland; A. Lilienfeld, Johns Hopkins University																	
LAB/BRANCH Office of the Director, Intramural Research Program																	
SECTION Neuroepidemiology Section																	
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																	
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.6	OTHER:															
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																	
SUMMARY OF WORK (200 words or less - underline keywords) This investigation is aimed (1) at evaluating the effect of <u>heart disease</u> and <u>hypertension</u> as potentially treatable <u>precursors</u> of <u>completed stroke</u> and <u>transient ischemic attacks</u> ; (2) at documenting unusual patterns of <u>cerebrovascular disease</u> ; (3) at determining the <u>autopsy patterns</u> for patients dying with cerebrovascular disease in a defined <u>community</u> ; and (4) at examining if <u>weather parameters</u> have any effect on stroke incidence. .																	

Project Description:

Objectives: To determine the following: (1) the risk of stroke and transient ischemic attacks in individuals with heart disease and/or hypertension as compared to the risk in individuals without these conditions; (2) whether the existence of pre-existing heart disease and/or hypertension affects the type of stroke and whether it affects survival following stroke; and (3) whether there is a particular time interval following the onset of heart disease or hypertension during which an individual is at high risk for stroke. In addition, other studies address the issue of the effect of weather on stroke incidence, and a description of autopsy findings for patients dying with stroke in a defined community.

Methods Employed: This first study involves a non-concurrent prospective approach evaluating a cohort of 2,000 elderly individuals. The type of analysis follows the person-years strategy and utilizes life-table methods. The investigations of weather variables and autopsy patterns are based on the records-linkage resource for residents of Rochester, Minnesota.

Major Findings: Transient ischemic attacks and stroke appear to have different risk factors; the previously published incidence rates for transient ischemic attacks are underestimates; and temperature has no effect on stroke incidence.

Significance: At the present time, there is little to suggest that improved medical management of the completed stroke will substantially affect the cerebrovascular disease problem. It would appear that greater benefit could be achieved by dealing with the precursors of stroke rather than delaying treatment until after the event has occurred. Previous studies of weather, showing a relationship to stroke mortality, were not confirmed when we examined incidence.

Proposed Course of Project: The forthcoming year will be devoted to data analysis and publication of the results.

Publications:

Rosenblatt, K.A., Whisnant, J.P., and Schoenberg, B.S.:
Temperature, snowfall, and the incidence of stroke:
Rochester, Minnesota, 1955-1969. Stroke, in press.

Schoenberg, B.S.: The epidemiology of ischemic cerebrovascular disease. In Portera-Sanchez, A. (Ed.): Cerebral Ischemia. Geneva, Excerpta Medica, in press.

Schoenberg, B.S.: Risk Factors for Cerebrovascular Disease. In Rose, F.C. (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02243-04 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>												
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Pediatric Neuroepidemiology</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: Bruce S. Schoenberg</td> <td style="width: 20%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td>Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) D. Schoenberg, M.S., Research Epidemiologist, Bethesda, Maryland; J.F. Mellinger, M.D., and M.R. Gomez, M.D., Department of Neurology, Mayo Clinic; B.W. Christine, M.D., M.P.H., Connecticut State Department of Health												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: <p style="text-align: center;">1.5</p>	PROFESSIONAL: <p style="text-align: center;">1.5</p>	OTHER:										
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS					
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER										
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) <p>The project documented the frequency of <u>primary intracranial neoplasms</u> in the <u>pediatric populations</u> of Rochester, Minnesota, and the State of Connecticut. In addition, we investigated the magnitude and risk factors for <u>cerebrovascular disease in infants and children</u> in the Rochester, Minnesota population. We have also initiated a study of the temporal trends in the incidence of cerebral palsy.</p>												

Project Description:

Objectives: To document 1) the frequency of primary neoplasms and cerebrovascular disease in pediatric populations; and 2) the temporal trends in the incidence of cerebral palsy. This had never been done before in a well-defined population group in the United States.

Methods Employed: Descriptive epidemiologic studies were carried out utilizing the resources of the Connecticut Tumor Registry and the Rochester, MN records-linkage system. In addition, a case-control study was undertaken to determine risk factors for perinatal intracranial hemorrhage.

Major Findings: The brain tumor incidence rate in children (<15 years of age) varied from 2.5-5.0 cases/100,000/year. The study demonstrated that neonatal intracranial hemorrhage is relatively common (1.1 cases/1,000 live births), that it is strongly associated with prematurity and hyaline membrane disease, and that it is difficult to recognize clinically. For pediatric cerebrovascular disease unassociated with birth, trauma, or infection, the incidence rate was 2.5/100,000/year. These cases were further characterized by survival, residual disability, and cause (whenever possible). The clinical and angiographic features of children with moyamoya disease were examined in detail. This condition appears to be more common than suggested by early case reports.

Significance: This study represents the first time that the magnitude of either brain tumors or cerebrovascular disease has been documented in a well-defined pediatric population.

Data from the study of cerebral palsy will be used to examine whether new developments in perinatal care affected the incidence of these conditions.

Proposed Course of Project: Additional diseases will be studied using similar methodology. A case-control study of cerebral palsy will follow the descriptive investigation. The problem of cerebrovascular disease in children will be re-evaluated following the introduction of computerized tomography as a diagnostic tool.

Publications:

Schoenberg, B.S.: Risk Factors for Stroke in Infants and Children. In Goldstein, M., Bolis, L., Fieschi, C., Gorini, S., and Millikan, C.H. (Eds.): Cerebrovascular Disorders and Stroke. New York, Raven Press, 1979, pp. 313-324.

Schoenberg, B.S., and Rose, F.C.: Clinical paediatric neuroepidemiology. Dev. Med. Child Neurol. 21: 677-680, 1979.

Schoenberg, B.S., and Schoenberg, D.G.: The Spectrum of Pediatric Cerebrovascular Disease. In Rose, F.C., (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02297-04 ODIR
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Mortality from Neurologic Disorders: National and International Comparisons		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Bruce S. Schoenberg Chief NES ODIR NINCDS		
COOPERATING UNITS (if any) W. Massey, M.D., Duke University; D.G. Schoenberg, M.S., Bethesda, Maryland		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) Although death certificate data are limited by possible misdiagnosis, incomplete case ascertainment, errors in coding, etc., detailed morbidity information on neurologic diseases for the entire U.S. and for other countries is not available. The Section has analyzed <u>mortality data for selected neurologic disorders</u> by country and by county in the U.S. The overall patterns which emerge may be useful in evaluating trends over time and in formulating etiologic hypotheses.		

Project Description:

Objectives: To analyze available mortality data by country and by county in the U.S.; to measure secular trends and formulate etiologic hypotheses.

Methods Employed: Age-adjusted death rates for selected neurologic disorders were calculated for 33 countries and for each U.S. county. Rates were ranked and patterns of mortality are being illustrated graphically and with maps.

Major Findings: Mortality from cerebrovascular disease has decreased in most developed countries over a 20-year period. This trend is not universal, however. For multiple sclerosis, countries initially reporting high mortality rates have generally reported declines while those with low rates earlier are reporting increases, so that the more recent mortality data for multiple sclerosis by country show less of a differential than previously observed.

Significance: Such detailed data are not available through sources of morbidity information. Consequently, we must utilize death certificate information to evaluate secular trends and patterns worldwide and by county within the U.S.

Proposed Course of Project: Analysis of these data is continuing, and publications will be prepared.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02299-04 ODIR										
PERIOD COVERED October 1, 1979 through September 30, 1980												
TITLE OF PROJECT (80 characters or less) Reviews of Epidemiologic Aspects of Neurologic Disease												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 15%;">Chief</td> <td style="width: 15%;">NES</td> <td style="width: 15%;">ODIR</td> <td style="width: 22%;">NINCDS</td> </tr> <tr> <td>Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) W. Massey, M.D., Duke University; D. Schoenberg, M.S., Bethesda, Maryland												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:										
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords) Development of new neurologic studies requires thorough historic and <u>methodologic reviews</u> of prior investigations. These yield important <u>unexplored etiologic clues</u> that may be investigated using current technology. Major emphasis has been given to <u>cerebrovascular disease</u> , <u>otitis media</u> , <u>inherited ataxias</u> , <u>Huntington's disease</u> , and <u>febrile seizures</u> .												

Project Description:

Objectives: To review comprehensively previous studies dealing with neurologic disorders in human populations, as well as other diseases with neurologic manifestations.

Methods Employed: Pertinent literature is critically reviewed for etiologic clues and unresolved issues which are answerable through properly designed neuroepidemiologic studies.

Major Findings: Suggested studies have been designed for cerebrovascular disease, otitis media, inherited ataxias, Huntington's disease, and febrile seizures. Some of these investigations are being pursued by the Section, as well as extramural scientists.

Significance: It is only through careful and critical review of previous efforts that a productive research program can be launched. These critical reviews are generally published so that both intramural and extramural investigators have access to this information.

Proposed Course of Project: These review efforts will continue and will generally focus on major neurologic diseases (e.g., dementia).

Publications:

Kudrjavcev, T.: Differential Diagnosis and Work-up. In Nelson, K.B., and Ellenberg, J.H. (Eds.): NIH Consensus Development Conference on Febrile Seizures. New York, Raven Press, in press.

Schoenberg, B.S.: The Epidemiologic Approach to Huntington's Disease. In Chase, T., Wexler, N., and Barbeau, A. (Eds.): Huntington's Disease. New York, Raven Press, 1979, pp. 1-11.

Schoenberg, B.S.: Evaluating the quality of medical research. South. Med. J. 73: 1-2, 1980.

Schoenberg, B.S.: Puzzling epidemics and neurochemical solutions: the interaction between neuroepidemiology and neurochemistry. In Rose, F.C. (Ed.): Metabolic Disorders of the Nervous System. Tunbridge, England, Pitman Medical, in press.

Schoenberg, B.S.: S. Weir Mitchell and the renaissance of early American neurology. In Rose, F.C., and Bynum, W.F. (Eds.): Historical Aspects of the Neurological Sciences. Tunbridge, England, Pitman Medical, in press.

Schoenberg, B.S., and Schoenberg, D.G.: Eponym: John
Abernethy. South. Med. J. 72: 1323-1324, 1979.

Schoenberg, D.G., and Schoenberg, B.S.: Eponym: Henry Bence
Jones. South. Med. J. 72: 605-606, 1979.

Schoenberg, D.G., and Schoenberg, B.S.: Eponym: Leo
Buerger. South. Med. J. 72: 737-738, 1979.

Schoenberg, D.G., and Schoenberg, B.S.: Eponym: Paget's
disease. South. Med. J. 72: 997-998, 1979.

Schoenberg, D.G., and Schoenberg, B.S.: Eponym: Sir Astley
Paston Cooper. South. Med. J. 72: 1193-1194, 1979.

Schoenberg, D.G., and Schoenberg, B.S.: Eponym: Theodor
Billroth. South. Med. J. 72: 1590-1591, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02300-04 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>												
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Clinical Course and Medical Care for Neurologic Disorders</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT												
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: F. Garcia-Pedroza</td> <td>Guest Worker</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: F. Garcia-Pedroza	Guest Worker	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: F. Garcia-Pedroza	Guest Worker	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) <p style="text-align: center;">J.P. Whisnant, Dept. of Neurology, Mayo Clinic, Rochester, Minnesota</p>												
LAB/BRANCH <p style="text-align: center;">Office of the Director, Intramural Research Program</p>												
SECTION <p style="text-align: center;">Neuroepidemiology Section</p>												
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>												
TOTAL MANYEARS: <p style="text-align: center;">1.5</p>	PROFESSIONAL: <p style="text-align: center;">1.5</p>	OTHER:										
CHECK APPROPRIATE BOX(ES)												
<table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS						
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)												
<p>The study uses a review and abstraction of data from records for a selected group of <u>neurological disorders</u>. It obtains the items of data necessary to determine onset of the disorder, duration, date and cause of death, or current status. These data will be used to construct <u>modified life tables</u> to estimate the <u>expectation of life after diagnosis</u>, the <u>survival curve</u> and <u>morbidity and severity estimates</u>. It will also include analysis of type and duration of <u>medical care</u> received by patients with neurologic disorders derived from a well-defined population.</p>												

Project Description:

Objectives: To determine the clinical course and patterns of medical care for all individuals with major neurologic disease in a given community.

Methods Employed: Cases of neurologic disease among residents of Rochester, Minnesota, have already been identified through previous studies. Medical encounters are documented through a records-linkage resource and this information has been abstracted.

Major Findings: The data for Rochester, Minnesota, residents with brain tumor or stroke have been collected and are being analyzed.

Significance: Rochester residents have access to high-quality medical care, and physicians with neurologic expertise are available within the community. Thus, the Rochester experience may provide some estimate of the pattern of medical care in the ideal situation in which the population has ready access to neurologic expertise, and in which there is little financial restraint to such care.

Proposed Course of Project: Data analysis and publication will follow.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02301-04 ODIR										
PERIOD COVERED October 1, 1979 through September 30, 1980												
TITLE OF PROJECT (80 characters or less) Collaborative Studies of Less Common or Less Debilitating Neurologic Disorders												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) M. Zack, M.D., Atlanta, Georgia; Neurosciences Program, WHO, Geneva, Switzerland; D. Duane, M.D., B. Sandok, M.D., Mayo Clinic												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 2.8	PROFESSIONAL: 1.9	OTHER: 0.9										
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords) A number of collaborative efforts involve the investigation of the characteristics of unusual or less debilitating (e.g., headache) neurologic disease phenomena. Unusual associations or <u>space/time clusters of neurologic disorders</u> may provide leads to etiology or therapy. These may be tested through more formal approaches.												

Project Description:

Objectives: To investigate and characterize unusual relationships, patterns, or phenomena associated with neurologic diseases.

Methods Employed: In collaboration with other governmental agencies (Center for Disease Control), international organizations (World Health Organization), and universities (Mayo Medical School) methods have been developed to investigate less common neurologic disorders. Several such studies have been completed. Current projects include investigation of space/time clusters of neurologic disease (with the Center for Disease Control), the development of survey strategies (with the World Health Organization and the Section on Disease Statistics Surveys), a study of myasthenia gravis and multiple sclerosis in the same patient (with the Mayo Clinic), and an investigation of neurologic disorders during pregnancy and the postpartum period (with the Mayo Clinic).

Major Findings: An unusual manifestation of aqueductal stenosis was reported. The magnitude of the Guillain-Barré syndrome was investigated in a well-defined population. This is only the second such study in the United States and provides a baseline for evaluating future phenomena (such as the increased occurrence with immunization against influenza A/New Jersey).

Proposed Course of Project: Several such ongoing studies will be analyzed and reported. New investigations are undertaken on an ad-hoc basis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02305-04 ODIR										
PERIOD COVERED October 1, 1979 through September 30, 1980												
TITLE OF PROJECT (80 characters or less) The Epidemiology of Intracranial Neoplasms												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) B.W. Christine, M.D., M.P.H., Connecticut State Dept. of Health; J.P. Whisnant, M.D., and R.J. Campbell, M.D., Mayo Clinic; L. Mahalak, M.D., Jackson, MS; A. Heck, M.D., Univ. of TN; R. Simon, M.D., Berkeley, CA; B. Jordan, B.A., Harvard Medical School												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:										
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS					
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER										
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) The Section has conducted extensive investigations on the descriptive <u>epidemiology of primary intracranial neoplasms</u> using data derived from population-based registries worldwide. Analytic studies were carried out to investigate the relationship between intracranial neoplasms and tumors occurring at other sites. These studies included careful review of tumor nomenclature, disease definitions, and survey strategies.												

Project Description:

Objectives: To resolve problems in nomenclature, disease definitions, and survey strategies; to define the magnitude and distribution of primary intracranial neoplasms; and to investigate the relationship between intra- and extracranial tumors.

Methods Employed: Descriptive epidemiologic techniques were applied to data obtained from tumor registries around the world. Wherever possible, cases were reviewed by the Section's staff. New analytic techniques for studies of multiple primary cancers were devised.

Major Findings: On the basis of the descriptive studies, two patterns of age-specific incidence emerged. Analyses of most population-based data worldwide revealed a small childhood peak, followed by a later peak between ages 50 and 80. Data for Rochester, Minnesota, however, showed the childhood peak, followed by an increasing incidence rate with increasing age. Careful study of this discrepancy showed 1) that the greater percentage of cases first diagnosed at autopsy in Rochester accounted in large part for this difference, and 2) that a substantial number of brain tumors remain undiagnosed in the elderly during life. Studies are currently underway to evaluate the role of computerized tomography in the diagnosis of brain tumors and to explain the recent increase in the incidence of pituitary tumors among women of child-bearing age. In addition, racial differentials in the frequency of certain intracranial tumors (meningiomas and pituitary adenomas) are being examined. Investigations of the relationship between intracranial neoplasms and extracranial tumors have been especially rewarding. An association was found between the occurrence of breast cancer and meningioma in women. This result raises interesting etiologic possibilities when considered with other evidence: 1) meningioma is the only common intracranial neoplasm with a higher incidence in females; 2) the abrupt clinical appearance or enlargement of this tumor during pregnancy has been described; and 3) the finding of estrogen receptor protein in meningioma has been reported. This has led to further work concerning estrogen receptors in other tumors such as malignant melanoma involving the nervous system.

Significance: Previous epidemiologic studies regarded all brain tumors as a single disease. The set of studies described above provided overwhelming evidence that the individual histologic types of primary intracranial neoplasms represent distinct disease entities. The analytic studies for the first time began to define risk factors for these tumors. Possible role of hormones in the etiology or growth of meningiomas may have therapeutic significance.

Proposed Course of Project: Several of these findings must still be prepared in a form suitable for publication.

Publications:

Annegers, J.A., Schoenberg, B.S., Okazaki, H., and Kurland, L.T.: Intracranial Neoplasms in Rochester, Minnesota, 1935-1977. In Rose, F.C., (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, in press.

Annegers, J.F., Schoenberg, B.S., Okazaki, H., and Kurland, L.T.: Effect of sources of case ascertainment on the incidence rates of primary intracranial neoplasms: Rochester, Minnesota, 1935-1977. Accepted for publication by the Archives of Neurology.

Schoenberg, B.S.: Cancer of Specific Tissues: Nervous System. In Schottenfeld, D., and Fraumeni Jr., J.F. (Eds.): Cancer Epidemiology and Prevention. Philadelphia, W.B. Saunders, in press.

Schoenberg, B.S., and Christine, B.W.: Malignant melanoma associated with breast cancer. Accepted for publication by the Southern Medical Journal.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02307-04 ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Educational Resources in Neurological Epidemiology</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <p>PI: Bruce S. Schoenberg Chief NES ODIR NINCDS</p>		
COOPERATING UNITS (if any) <p>D. Schoenberg, M.S., Research Epidemiologist, Bethesda, Maryland</p>		
LAB/BRANCH <p>Office of the Director, Intramural Research Program</p>		
SECTION <p>Neuroepidemiology Section</p>		
INSTITUTE AND LOCATION <p>NINCDS, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">1.5</p>	PROFESSIONAL: <p style="text-align: center;">1.5</p>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS </div> <div> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p>A series of four <u>videotapes</u> on the principles of neuroepidemiology were produced by the Section. A two-day international <u>conference</u> on neuro-epidemiology was held in 1977; a one-day <u>course</u> was held in 1977; a one-day symposium was held in 1979; a 1-1/2 day <u>course</u> will be held in Asia in 1980; a one-week course will be held in Europe in 1981; a one-day symposium will be held in Europe in 1981; a one-day symposium will be held in Asia in 1981; and a one-week course is planned for Africa in 1981. A textbook entitled <u>Neurological Epidemiology: Principles and Clinical Applications</u> was published during 1978.</p>		

Project Description:

Objectives: The severe shortage of available manpower in neuroepidemiology necessitated development of an educational program which was initiated by the Section.

Methods Employed: A series of four videotapes were produced by the Section and are distributed on a loan-basis without charge - the 1977 course and conference were held in cooperation with Georgetown University. The 1979 Symposium was held in conjunction with Yale University. A textbook on neurological epidemiology was published by Raven Press in 1978.

Major Findings: Attendance at the conferences included over 250 representatives from Asia, Africa, Europe, Latin America, and the U.S. Approximately 2000 copies of the textbook have been requested.

Significance: With such a limited supply of expertise in neurological epidemiology, these educational resources fill an important need in the neurosciences.

Proposed Course of the Project: Further courses and additional videotapes have been requested. Furthermore, videotapes are being converted for use in Europe and Asia. The Section has been requested by the World Health Organization to organize the following courses in neuroepidemiology: a 1-1/2 day course to be held in Asia in 1980; a one-week course in Europe in 1981; and a one-week course in Africa in 1981. In addition, there will be a one-day symposium in conjunction with the meeting of the International Epidemiological Association in Europe during 1981 and a one-day symposium in conjunction with the World Congress of Neurology during 1981 in Kyoto, Japan.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02369-02 ODIR
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Cigarette Smoking and Parkinson's Disease		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; padding: 10px 0;"> PI: Robert J. Baumann Neurologist NES ODIR NINCDS </div>		
COOPERATING UNITS (if any) University of Kentucky		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">2.5</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">2.0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; padding: 5px 0;"> <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; padding: 5px 0;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p>This is a case-control study of cigarette smoking and other factors associated with <u>Parkinson's disease</u>. This project has been completed.</p>		

Project Description:

Objectives: To re-investigate the negative association between cigarette smoking and Parkinson's disease reported by others.

Methods Employed: A case-control study was employed.

Major Findings: The investigation confirmed previous reports of an inverse relationship (i.e., a low rate of smoking among those with Parkinson's disease as compared to the smoking rate among controls).

Significance: Previous studies found fewer cigarette smokers among persons with Parkinson's disease than among other patients. This study reconfirmed this finding using non-patient controls. Possible explanations include selective mortality, premorbid behavioral or constitutional attributes, the presence of an anti-parkinsonian chemical in cigarette smoke, or some combination of these factors.

Proposed Course of Project: This project has been completed. The results have been prepared for publication during this fiscal year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02370-02 ODIR										
PERIOD COVERED October 1, 1979 through September 30, 1980												
TITLE OF PROJECT (80 characters or less) Racial Differentials in the Prevalence of Major Neurologic Disorders												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Dallas Anderson</td> <td>Survey Statistician</td> <td>OBFS</td> <td>OD</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Dallas Anderson	Survey Statistician	OBFS	OD	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Dallas Anderson	Survey Statistician	OBFS	OD	NINCDS								
COOPERATING UNITS (if any) Office of Biometry and Field Studies, OD, NINCDS; A. Haerer, M.D., University of Mississippi; U.S. Bureau of the Census												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 20.0 7.0	PROFESSIONAL: 5.0 4.0	OTHER: 15.0 3.0										
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this study is to accurately document possible <u>racial differentials</u> in the prevalence of <u>major neurologic disorders</u> by surveying an entire county, with a biracial population of approximately 25,000. The disorders investigated include <u>cerebral palsy</u> , <u>dementia</u> , <u>psychomotor delay</u> , <u>epilepsy</u> , <u>Parkinson's disease</u> , and <u>cerebrovascular disease</u> .												

Project Description:

Objectives: To accurately document possible racial differentials in the prevalence of major neurologic disorders in a well-defined biracial population.

Methods Employed: A strategy was developed which eliminated the requirement that persons must have entered the health care system for detection of disease. The basis of the investigation was a door-to-door survey which utilized a detailed questionnaire inquiring not only about diagnoses, but also about signs and symptoms suggestive of neurologic dysfunction. Over 99% of the households agreed to the interview. Those household members suspected of having one of the disorders of interest were then asked to have a neurologic examination conducted by a senior, board-certified neurologist.

Major Findings: The data are currently being analyzed, and no findings are yet available. There have been delays in obtaining tabulations from the U.S. Census Bureau, because of the heavy demands of the 1980 U.S. Census.

Significance: A number of early investigations suggested possible differences in neurologic disease frequency by race, but were based on hospital or clinic experience and these studies could not identify a well-defined population from which cases were derived. Population-based studies followed, but questions concerning the results centered on possible racial differentials in access to expertise in neurologic diagnosis and treatment. The present study should eliminate these potential sources of bias.

Proposed Course of Project: The interviews and examinations have been completed, and the data are being edited and analyzed. Similar strategies are being developed for application in developing countries, in collaboration with the World Health Organization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02423-01 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>												
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Development of Data Resources for Neuroepidemiology</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 11%;">NES</td> <td style="width: 11%;">ODIR</td> <td style="width: 11%;">NINCDS</td> </tr> <tr> <td>Rudy Capildeo</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Rudy Capildeo	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Rudy Capildeo	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) F. Clifford Rose, M.B., F.R.C.P., B. Benjamin, Ph.D., S. Haberman, M.A., F.I.A., Charing Cross Neuroepidemiology Unit, London, England; W. Sibley, M.D., Univ. of Arizona, Tucson, Arizona; and R. Katzman, M.D., Albert Einstein Sch. of Med., New York, New York												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: <p style="text-align: center;">1.0</p>	PROFESSIONAL: <p style="text-align: center;">1.0</p>	OTHER:										
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SUMMARY OF WORK (200 words or less - underline keywords) <p>To develop 1) a <u>registry of hospitalized patients with neurologic diseases</u> in a well-defined population of 30.5 million people, and 2) <u>resources for case-control studies of multiple sclerosis and Alzheimer's disease</u> using <u>uniform methods of data collection</u>.</p>												

Project Description:

Objectives: To develop a functioning registry of hospitalized patients with neurologic disease in a well-defined population of 3.5 million inhabitants. The project also involves the establishment of resources for case-control studies of multiple sclerosis and Alzheimer's disease, utilizing a uniform data base for each disorder.

Methods Employed: Based on information routinely collected by the British National Health Service, a registry is being established for all neurologic inpatients derived from a population of 3.5 million (North-West Thames Metropolitan Region, London, England). Recorded data includes demographic information, length of hospitalization, details of surgical procedures, and up to five diagnoses. The registry data are being tested for accuracy of diagnosis and coding, hospital and physician cooperation, completeness of case ascertainment, and delay from hospital discharge to data entry in the registry. The Registry's operation will be investigated with studies of the Guillain-Barré syndrome and primary intracranial neoplasms.

For the establishment of resources for case-control studies, a uniform data base is being established for collaborative studies of multiple sclerosis and Alzheimer's disease.

Major Findings: The initial numbers of cases of the Guillain-Barré syndrome and primary intracranial neoplasms are in line with what would be expected on the basis of U.S. statistics.

Significance: An important problem for the neuroepidemiologist is the enormous cost of maintaining neurologic surveillance on a large number of patients and the long period of time generally required to investigate temporal patterns. The establishment of a registry of neurologic diseases based on an ongoing government-supported collection of data, will allow neuroepidemiologists to respond rapidly to etiologic questions at minimal cost. The use of a uniform data base for case-control studies will permit the pooling of information from several centers, thereby facilitating the examination of relatively uncommon phenomena.

Proposed Course of Project: Attempts will be made to improve the efficiency of the Registry's operation and to include data concerning outpatients as well.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02424-01 ODIR										
PERIOD COVERED October 1, 1979 through September 30, 1980												
TITLE OF PROJECT (80 characters or less) Standardized Nomenclature and Coding of Neurologic Diseases												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Rudy Capildeo</td> <td style="width: 33%;">Neurologist</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Bruce S. Schoenberg</td> <td>Chief</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Rudy Capildeo	Neurologist	NES	ODIR	NINCDS	Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS
PI: Rudy Capildeo	Neurologist	NES	ODIR	NINCDS								
Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) F. Clifford Rose, M.B., F.R.C.P., B. Benjamin, Ph.D., S. Haberman, M.A., F.I.A., Charing Cross Neuroepidemiology Unit, London, England; and L. Schut, M.D., Minneapolis, Minnesota												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
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SUMMARY OF WORK (200 words or less - underline keywords) To develop an internationally acceptable <u>standard</u> of <u>nomenclature</u> , <u>classification</u> , and <u>coding</u> of <u>neurologic disorders</u> .												

Project Description:

Objectives: To develop an internationally acceptable standard of nomenclature, classification, and coding of neurologic disorders.

Methods Employed: The new system of classification and coding first follows a thorough review of existing schemes, with particular attention to deficiencies. The resulting new system will attempt to correct these deficiencies and aim to facilitate compliance by physicians.

Major Findings: A review of the 9th revision of the International Classification of Diseases reveals that it is seriously deficient with regard to neurologic diseases. Disorders with different etiologies and different epidemiologic patterns are categorized together. Neurologic diseases may be classified under endocrine or psychiatric conditions.

Significance: Epidemiologic studies have two basic requirements: uniformity and accuracy of data collection. This necessitates the use of a standardized, internationally acceptable classification and coding scheme that can be easily used by physicians, regardless of the available medical facilities.

Proposed Course of Project: Pilot studies will be carried out to test and revise the new classification and coding scheme. This will be followed by periodic meetings to consider the adaptation of this system. The final results will be presented to the World Health Organization for possible incorporation into their own International Classification of Diseases.

Publications:

Capildeo, R., Haberman, S., and Rose, F.C.: The Classification and Coding of Neurological Disease. In Rose, F.C. (Ed.): Progress in Neuroepidemiology. Tunbridge, England, Pitman Medical, 1980.

Capildeo, R., Haberman, S., and Rose, F.C.: The Classification of Parkinson's Disease. In Rose, F.C. and Capildeo, R. (Eds.): Progress in Parkinson's Disease Research. Tunbridge, England, Pitman Medical, in press.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Section on Neurotoxicology, ODIR
National Institute of Neurological and Communicative Disorders and Stroke

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Annuual Report
for Period October 1, 1979, through September 30, 1980
Neurotoxicology Section
Office of the Director
Intramural Research Program
National Institute of Neurological and Communicative
Disorders and Stroke
Ellen K. Silbergeld, Chief

SUMMARY

1. Neuroendocrine Alterations in Lead Exposure

Prolactin levels in lead-exposed rats are being studied in collaboration with the Pharmacology Section, Experimental Therapeutics Branch. Initial results suggest that resting prolactin levels are higher in lead-exposed animals as compared to controls. This is consistent with the hyperreactivity of lead-exposed animals and may be correlated with alterations in central dopaminergic and GABAergic neurochemistry.

2. Neurochemical Markers for Lead Exposure in Children

We demonstrated earlier, in both rats and children, an association between low level lead exposure and increased release of dopamine. Continuing clinical studies now show that the increased urinary excretion of homovanillic acid is significantly and positively correlated with concentrations of lead in blood of children 0.5-3 years old. This study is being conducted in collaboration with the John F. Kennedy Institute and the Department of Pediatrics, Johns Hopkins Medical School, Baltimore, Maryland.

3. Lead, Heme, and GABA

Experimental lead exposure is associated with inhibition of GABA release and a concomitant supersensitivity of postsynaptic GABA receptors. Since this action of lead cannot be reproduced in vitro, indirect actions of the heavy metal (when administered in vivo) may be involved. We have explored the possible role of altered heme synthesis in the antiGABAergic effects of in vivo lead exposure. The hypothesis is supported by the following findings:

- (1) succinyl acetone (which acts to block the same enzymatic step as lead in the heme synthetic pathway) also inhibits GABA release after in vivo administration, and
- (2) administration of hematin reverses lead-induced decreases in heme synthesis and appears to counteract the effects of lead on GABAergic function measured both neurochemically and behaviorally.

These studies have been conducted partially in collaboration with the Metabolism Branch, NCI, and the Department of Pharmacology, USUHS.

4. Manganese and Aging

Manganese (chronic, high level exposure) is known to cause a syndrome possibly identical to idiopathic parkinsonism. Because of the increased incidence of idiopathic parkinsonism in aged persons, studies are being conducted on possible special susceptibility of aged animals. Aged animals are more sensitive to the adverse effects of manganese on locomotion behavior.

5. Neurotoxicity of cis-Platinum

Clinical exposure to platinum can occur in the course of cis-platinum therapy for certain cancers. Since neurotoxic side-effects have been described, we have studied the effects of cis-platinum exposure on motor behavior in rats, in collaboration with the Department of Neuropathology, Johns Hopkins Hospital. Intoxicated rats show alterations in gait, measured by quantitative methods developed previously in the Section. These effects are observed before overt neuropathological signs and may be useful in monitoring unwanted side effects as well as in determining sites of action of platinum.

6. Hormones and Extrapyramidal Function

Increased circulating levels of estrogens can result from the use of synthetic estrogens in food production and in pharmacotherapy, from the side-effects of neuroleptic therapy, and from environmental exposure to such compounds as kepone, and the polyhalogenated hydrocarbons PCB and PBB. Male rats, treated once with estrogen or PBBs, develop supersensitive dopamine receptors in striatum. Supersensitivity can be demonstrated neurochemically and behaviorally. Estrogen-induced dopamine receptor supersensitivity is dependent upon a pituitary factor, since hypophysectomy completely blocks this response to estrogen administration. The factor may be prolactin, whose secretion is increased by estrogen. Administration of prolactin to male rats also increases dopamine receptors.

7. Prolactin and Neuroleptic-induced changes in Dopamine receptors

The increased dopamine receptor sensitivity produced by long term neuroleptic administration may also importantly involve the pituitary, since hypophysectomized rats fail to demonstrate supersensitivity after 3 weeks' treatment with haloperidol. These are among the first extrahypothalamic effects reported for estrogen and prolactin in the CNS.

8. Kepone and Neuroendocrine Function

After neonatal exposure to kepone, rats have high levels of circulating estrogens and demonstrate increased behavioral responses to amphetamine. However, these effects are not clearly dose-related to kepone, and may involve other, nonhormonal effects of the pesticide. These studies were conducted in collaboration with the Laboratory of Neurobehavioral Toxicology, NIEHS.

9. Neurotoxic Actions of Erythrosin B

Erythrosin B, (Red No. 3) is an artificial food dye of the fluorescein or xanthene family. We reported earlier that it can block synaptosomal dopamine uptake. This action appears to reflect its highly specific and very potent action as an inhibitor of Na,K-ATPase. Of the structural analogs of erythrosin B investigated, only the dyes rose bengal and eosin Y, and the derivative di-iodofluorescein are active as ATPase inhibitors. Erythrosin B is equipotent to ouabain. However, unlike ouabain, erythrosin B specifically inhibits the Na,K-ATPase identified as unique to brain tissue; it is being investigated as a ligand for use in purifying and characterizing this enzyme.

10. Factors Related to Variable Sensitivity to Neurotoxins

A significant public health problem in toxicology is the existence of specially sensitive groups requiring increased protection from exposure to toxic compounds. Pharmacogenetics are important determinants of such susceptibility. Differences were defined among 20 rats strains in terms of numbers of dopamine receptors and muscarinic cholinergic receptors,

activity of type A and type B monoamine oxidases, brain Na,K-ATPase, and heme-dependent drug metabolizing enzymes. In collaboration with the Department of Pharmacology, USUHS, these differences are being exploited to study special susceptibility to artificial food dyes, heavy metals, and other toxins.

Increased sensitivity to environmental toxins may also result from perinatal experiences. The fetal alcohol syndrome is a model for early exposure resulting in compromised ability of the nervous system to withstand later neurotoxin exposure. Some of the actions of ethanol may result from its effects on ligand binding to dopamine receptors and its dose-dependent ability to inhibit and then stimulate ATPase.

11. Neurochemical Bases for Scrapie

In collaboration with the Laboratory of Central Nervous System Studies, it was found that scrapie-infected hamsters develop a dramatic hypersensitivity to serotonin agonists and precursors. Scrapie infection may therefore cause a specific disturbance to serotonergic pathways before inducing a widespread degeneration of the CNS.

12. Analytic Electron Microscopy

Lead can rapidly enter both nerve terminals and isolated capillary endothelial cells. This uptake process is not readily reversible after lead is bound to intracellular sites within mitochondria in both tissues. In collaboration with the Department of Neurology, Tufts Medical School, and the Department of Neuropathology, Johns Hopkins Hospital, investigations are underway on the localization and identification of heavy metals in nervous tissue obtained postmortem from patients exposed to cis-platinum for chemotherapy of cancer and from patients with amyelotrophic lateral sclerosis and Alzheimer's disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 02319-03 ODIR										
PERIOD COVERED October 1, 1979 to September 30, 1980												
TITLE OF PROJECT (80 characters or less) Analytic Electron Microscopy in Neurochemistry												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Ellen K. Silbergeld,</td> <td style="width: 15%;">Chief</td> <td style="width: 10%;">NTS</td> <td style="width: 9%;">NINCDS</td> </tr> <tr> <td>Others:</td> <td>C. Fiori</td> <td>Physicist</td> <td>BEIB</td> <td>NIH</td> </tr> </table>			PI:	Ellen K. Silbergeld,	Chief	NTS	NINCDS	Others:	C. Fiori	Physicist	BEIB	NIH
PI:	Ellen K. Silbergeld,	Chief	NTS	NINCDS								
Others:	C. Fiori	Physicist	BEIB	NIH								
COOPERATING UNITS (if any) Department of Neuropathology, Johns Hopkins Hospital, Baltimore MD; Department of Neurology, Tufts Medical School, Boston MA; Department of Neurology, Univ. of Michigan Medical School, Ann Arbor, MI; BEIB, NIH.												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Section on Neurotoxicology												
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SUMMARY OF WORK (200 words or less - underline keywords) Recent technological advances in coupling <u>electron microscopy</u> to analytic techniques involving electron-electron interactions have been applied to <u>neuro-chemistry</u> and neuropathology. Localization of <u>heavy metals</u> was successfully studied at the subcellular level in experimental <u>lead poisoning</u> , <u>cis-platinum</u> treatment, and <u>manganese</u> exposure. Clinically, brain tissue from patients with <u>Alzheimers disease</u> and <u>amyelotrophic lateral sclerosis</u> was studied for increased local concentrations of <u>aluminum</u> , <u>lead</u> , and other toxic metals. Metabolism of endogenous elements, <u>elemental drugs</u> (lithium) and tagged neurotransmitters and receptor ligands is also being studied.												

Project DescriptionObjectives:

- (1) to determine the applicability of new techniques in analytic electron microscopy (x-ray microprobe analysis and electron energy loss spectroscopy) to studying neurochemistry and neurotoxicology.
- (2) to determine local concentrations of toxic metals in brain tissue in relation to possible sites and mechanisms of action in the CNS.

Methods employed:

Analytic electron microscopy exploits electron-electron events occurring when specimens containing elements and molecules are bombarded with the x-ray beam used to produce images in scanning-transmission or transmission electron microscopy (STEM or TEM). Using new instrumentation developed at the Biomedical Engineering and Instrumentation Branch, NIH, both clinical and experimental tissues have been examined. A critical problem in such studies is tissue preparation, since at least two factors can interfere with analysis. One is the addition of exogenous compounds during tissue preparation for preparation for conventional electron microscopy (osmium, lead, and uranyl compounds, used in staining and fixation); the other is the loss of endogenous compounds during fixation, embedding and analysis (particularly diffusible ions). Several types of preparations are used to compare the impact of these two factors: rapidly air-dried homogenates and purified fractions of subcellular components; and variations of conventional and freeze-substituted preparations of thin-sectioned material. In collaboration with the Department of Neuropathology, Johns Hopkins, and the Department of Neurology, Tufts Medical School, we have examined conventionally prepared material from human autopsy representing several diseases in which abnormal metabolism of metals has been hypothesized to play a part, either as etiology or pathology.

Results etc.

Earlier studies demonstrated that in experimental animals, lead can enter neurons rapidly and irreversibly. This phenomenon was also observed in capillary endothelial cells isolated from rats and exposed in vitro to lead. Exposure to lead produces peculiarly electron-dense intramitochondrial inclusions. These granules also contain large amounts of calcium in close spatial association with lead, which suggests a co-precipitation of lead and calcium into the matrix of the mitochondrion. The increased accumulation of calcium by mitochondria of lead-poisoned capillaries is similar to biochemical observations we have made in neurons. Lead also increases the uptake of calcium into isolated brain capillaries, shifts the affinity for the uptake of calcium by mitochondria to higher concentrations of calcium, and inhibits the efflux of calcium from synaptosomal mitochondria. The functional significance of an apparently selective intramitochondrial sequestration of lead in capillaries has implications for cellular and blood-brain barrier processes. An alteration in endothelial cell calcium metabolism may be associated with disruptions in fluid and electrolyte transport across the brain capillary wall and, as a consequence, with the development of brain edema in acute lead

poisoning in vivo. This study also demonstrates that x-ray microprobe analysis in STEM can provide spatial information with a resolution on the order of 10 nm.

Studies were conducted on localization of manganese in caudate tissue from rats exposed neonatally to the metal. Manganese was detected only rarely, and exclusively in association with cell membranes. However, these rats expressed very little behavioral signs of neurotoxic effects.

Tissue was examined from patients with Alzheimers disease and amyelotrophic lateral sclerosis (ALS), in collaboration with the Department of Neurology, Tufts Medical School. Some tissue appeared to contain high levels of aluminum in myelinated fibers of the spinal cord (Alzheimers); however, problems of elemental contamination may compromise these results. No notable subcellular concentrations of lead were found in ALS specimens, which argues against some speculations of an etiology of earlier lead exposure in this disease.

Studies on localization of tagged transmitters and receptor ligands (diFl-serotonin, I-bungarotixin, flunitrazepam) have been unsuccessful. The data suggest that either (1) preparation procedures remove loaded transmitters and receptor ligands or (2) the concentration of these compounds within a defined subcellular volume is below detection limits for present instrumentation.

Proposed course

Further studies are planned for examination of postmortem tissue from several neurological disorders in which increased local concentrations of metals in hypothesized to occur. We will use preparations enriched in specific neurotransmitter receptors in order to determine whether pre- and postsynaptic binding sites can be distinguished. The techniques of analytic electron microscopy will be employed in our ongoing studies of the mechanisms of action of the neurotoxins platinum and manganese.

Publications:

Silbergeld, E.K., Wolinsky, J., Goldstein, G.W.: Electron probe microanalysis of isolated brain capillaries poisoned with lead. Brain Res. 189: 369-376, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02264-04 ODIR
PERIOD COVERED October 1, 1979, to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Animal Models of Neurological Disease		
NAMES, LABORATORY AND INSTITUTL AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Ellen K. Silbergeld Other: Robert E. Hurska Sally M. Anderson Mark De Ryck Stephen J. Morris Hugh A. Tilson Steven Cohen Roger Weir Eugenio Parati	Chief Staff Fellow Expert Consultant Visiting Fellow Expert Consultant Pharmacologist IPA Guest Worker Visiting Fellow	NTS NTS NTS NTS NTS LNBT NTS NTS NTS ETB
NINCDS NINCDS NINCDS NINCDS NINCDS NIEHS NINCDS NINCDS NINCDS		
COOPERATING UNITS (if any) Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD; Division of Hematology Research, Scripps Clinic, La Jolla, CA; Department of Neuropathology, Johns Hopkins Hospital, Baltimore, MD; Dept. of Pharmacology, USUHS, Bethesda, MD; LNBT, NIEHS; ETB, NINCDS.		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Section on Neurotoxicology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 6.0	PROFESSIONAL: 4.4	OTHER: 1.6
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p> This project has concerned the development of <u>animal models</u> of neurological disease produced by exposure to synthetic and <u>naturally occurring neurotoxins</u> in the <u>environment</u>. The interaction of various toxins and <u>neurotransmitters</u> and <u>hormones</u> in the CNS have provided the focus for combined <u>behavioral and neurochemical</u> studies emphasizing basic mechanisms of action of proposed <u>neurotoxins</u>. Major topics studied this past year were: (1) effects of <u>heavy metals</u> on neurotransmitter and neuroendocrine function; (2) effects of <u>estrogens</u> on basal ganglia function; (3) effects of <u>artificial food colors</u> on neuronal membranes and neurotransmission; (4) <u>genetically</u> determined variations in neurotoxicity. </p>		

Project DescriptionObjectives:

(1) To develop animal models of neurological disease caused by environmental neurotoxins, and (2) to explicate mechanisms of action of these neurotoxins at specific sites in the nervous system.

A. Heavy MetalsMethods employed:

Chronic and acute in vivo, as well as in vitro, exposure paradigms are used to study the effects of lead, manganese, and cis-platinum on neurochemistry and behavior. Neurochemical studies have concentrated upon GABAergic-dopaminergic interactions (lead and manganese), with special studies on hypothalamic-pituitary neuroendocrine effects. Behavioral studies have concentrated on effects of lead on seizure sensitivity and effects of manganese and cis-platinum on motor function, using techniques for the quantitative assessment of motor function developed over the last year.

Major findings and the Significance to Biomedical Research and the Program of the Institute:1. Lead Poisoning:

Lead (in vitro and in vivo) is able to increase the stimulated release of dopamine from nerve terminals in the CNS. This increased release can be peripherally monitored in animals and children by measuring the dopamine metabolites homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) in urine. A series of clinical studies on over 70 children, conducted in collaboration with the Department of Pediatrics, Johns Hopkins Medical School, Baltimore, Maryland, has been completed in 1980. Children with undue lead exposure excrete significantly increased amounts of HVA. These increases in HVA are positively correlated with increased concentrations of lead in blood ($>30\mu\text{g}/100\text{ ml}$) but not correlated with any indicators of lead-induced inhibition of heme biosynthesis. In nine children, restudied after chelation therapy, urinary excretion of HVA appeared to decline towards normal levels along with declining levels of lead in their blood. The data provide the first evidence in humans for a dose-effect relationship between blood lead concentrations and a biochemical indicator of an effect of lead on neurochemistry. We are investigating the possibility of measuring plasma pituitary hormones (growth hormone and prolactin) as indices of lead neurotoxicity in these asymptomatic children. Initial experiments conducted in collaboration with the Pharmacology Section, Experimental Therapeutics Branch, NINCDS, suggest that lead exposure of neonatal rats is associated with increased resting levels of plasma prolactin.

Lead exposure, in animals, is also associated with decreased release of GABA and increased sensitivity to convulsants (GABA antagonists). These effects appear rapidly, as soon as four days after initiation of lead exposure, but cannot be produced when lead is added in vitro to appropriate

preparations of CNS tissue. The results suggest that a factor other than lead may be involved in the anti-GABAergic effects associated with in vivo exposure. Such a factor may be one of the increased porphyrin precursors which accumulate as a consequence of lead-induced inhibition of heme synthesis. Lead inhibits heme synthesis by blocking the enzyme or aminolevulinic acid dehydratase, with the result that the precursor aminolevulinic acid (ALA) accumulated 30-70x normal levels (measured in urine). ALA is an antagonist of GABA binding to its postsynaptic (and possibly also presynaptic) GABA receptors. Aspects of heme synthesis in acutely and chronically lead-exposed animals were studied in collaboration with the Department of Pharmacology, USUHS; the Metabolism Branch, NCI, and the Division of Hematology, Scripps Clinic, La Jolla, CA. Unlike other types of porphyrinopathies, experimental lead poisoning is not associated with a derepression-type induction of the enzyme ALA synthase. Lead-induced increases in ALA accumulation can be blocked by administration of hematin; hematin also appears to counteract the GABAergic effects of lead on transmitter release and behavioral sensitivity to convulsants.

2. Manganese

Exposure of neonatal rats to relatively low levels of manganese chloride in drinking water or diet does not produce any overt neurotoxic signs. The resistance of the young animal may reflect the essentiality of manganese as a trace element in neurological development. Exposure of aged rats to low levels of manganese is more toxic. Toxicity is first manifested neurochemically in changes in dopamine uptake (reduced) and activity of the enzyme glutamic acid decarboxylase (increased). These effects are consistent with a parkinsonian-like behavioral neurotoxicity associated with manganese intoxication.

3. cis-Platinum

Rats were treated with cis-platinum under paradigms analogous to those used in treatment of cancer in humans. In collaboration with the Department of Neuropathology, Johns Hopkins Medical School, we have studied these intoxicated animals for behavioral signs of neurological dysfunction. Platinum-treated rats demonstrated abnormalities of movement and gait, as defined by our methods for quantitative assessment of motor function in rodents.

Projected Course:

Studies on lead will continue in order to define the role of altered heme synthesis in producing neurotoxic signs. Other means of disrupting heme synthesis, such as succinyl acetone, without increasing body lead burdens, will be used to investigate whether similar effects on behavior and neurochemistry result from similar biochemical perturbations. Studies will continue to determine if hematin reversal of changes in heme synthesis can ameliorate changes in CNS GABA function and GABA-dependent behavior. Alterations in neuroendocrine parameters will be further defined, particularly with respect to pituitary or hypothalamic GABA receptors involved in the

control of hormone secretion. Also the utility of measuring plasma hormones as indicators of lead intoxication will be explored in a pilot study in collaboration with Johns Hopkins. Correlations between urinary catecholamines and plasma hormone measurements will be made to determine dose-effect relationships and reversibility.

The first full studies on the effects of long-term, low-level manganese exposure in aging rats will be concluded in the fall of 1980. Studies will produce information on dopaminergic, cholinergic, GABAergic, and neuroendocrine function in both manganese-exposed and control animals aged 2-1/2 years. Neuropathology and motor function in aged rats and aged rats exposed to manganese will also be assessed.

Further studies on cis-platinum exposed rats will be conducted, to correlate neurobehavioral dysfunction with neuropathological changes in the spinal cord and cerebellum. Reversibility of platinum-induced changes in motor function will be investigated.

B. Synthetic Estrogens and Estrogenic Toxins

Methods employed:

Rats are treated with synthetic estrogens or the estrogenic pesticide kepone or a polybrominated biphenyl mixture (Aroclor mixture from NIEHS). Both male and female rats are used, and treatment paradigms vary from prenatal exposure (to model exposure to diethylstilbestrol used to induce abortion and to prevent miscarriage at different times during gestation) to subacute exposure of adult animals (to model exposure resulting from use of estrogens in food production and in pharmacotherapies, as well as from environmental contamination). Neurochemical effects are assessed by studying neurotransmitter metabolism and synaptic membrane receptors. Behavioral effects are studied using special preparations (such as the rat with a unilateral lesion of the nigrostriatal dopamine pathway) and standard behavioral assays for dopamine receptor stimulation. Hormonal effects of estrogen administration are monitored by measurement of plasma prolactin.

Major Findings etc.

Adult, male rats treated once subcutaneously with estradiol valerate develop significant increases in the number of striatal dopamine receptors. This does not occur in female rats. Other striatal receptors are not affected; however, the activity of glutamic acid decarboxylase in both striatum and substantia nigra appears to be increased. Only the physiologically active beta-diastereomer produced this increase in receptor number. Neurochemical supersensitivity was correlated with increases in behavioral response to dopaminergic receptor stimulation, monitored in studies of rotation and stereotypy. In collaboration with the LBNT, NIEHS, we found similar changes produced by treating rats with kepone and PBBs. The neurological dysfunctions described in persons environmentally exposed to these compounds may be related to the activity of these environmental contaminants to increase levels of estrogens.

The mechanisms of the striatal effect of estrogen have been investigated. No effects of estrogen were observed in vitro. Increases in dopamine receptors were observed four days after subcutaneous administration. This delayed time course and the lack of in vitro activity both support the hypothesis that these effects are dependent upon protein synthesis, related to genomic effects of the hormone. Further, our research indicates that the striatal results of estrogen administration may be indirect. Removal of the pituitary prevents estrogen-induced dopamine receptor supersensitivity, measured neurochemically, and increased behavioral responses to dopamine agonists. The pituitary factor which transduces estrogen effects may be prolactin.

The relevance of these results to neurological disease was explored by investigating an important clinical syndrome associated with increased dopamine receptor sensitivity, the dyskinesia produced by chronic administration of neuroleptic drugs. Haloperidol administration increases prolactin secretion from the pituitary, probably by blocking dopamine acting as the prolactin inhibitory factor. We have found that hypophysectomy prevents the increase in striatal dopamine receptors occurring in rats after three weeks treatment with haloperidol. These results serve to indicate an important role of pituitary hormones in basal ganglia function and, as such, are among the first reports of extrahypothalamic actions of estrogen, and possibly prolactin, in the CNS.

Projected Course:

Other models of exposure relevant to human hyperprolactinemia and increased estrogen levels will be studied. Further experiments will be conducted to define the nature of the participation of the pituitary in the observed effects of estrogen on striatal dopamine and GABA functions. The role of pituitary and gonadal hormones in sexual differentiation of the brain and drug-induced changes in receptor function (agonist and antagonists) will be further studied. We will continue work on the nature of sex differences in response to estrogen, prolactin, and estrogenic neurotoxins such as kepone.

C. Neurotoxic Actions of Artificial Food Colors

Methods:

In order to study in vitro action of artificial food dyes, several preparations of CNS and peripheral tissues have been used. Synaptic membranes (purified and crude) are used in assays of ligand binding (^3H -ouabain and ^3H -digitoxin); synaptosomes (purified) are used in studies of ion fluxes, utilizing ^{22}Na for Na and ^{86}Rb for K; brain homogenates are used for studying catalytic activity of ATPase (production of Pi from ATP). Red cell ghosts were prepared from human and rat blood to study the enzyme Na,K-ATPase from a nonneural source.

Major Findings:

The halogenated fluorescein dyes are widely used in foods and cosmetics. One of these compounds, erythrosin B (tetraiodofluorescein), or Red No. 3, is

neuroactive in vitro as an inhibitor of neurotransmitter uptake by isolated nerve terminals. Investigations of this demonstrate that the inhibition is noncompetitive and confined to sodium-dependent processes; in the case of glutamate uptake, this effect can be defined as a shift in the stoichiometric co-transport relationship between Na and glutamate. A major mechanism for regulating sodium metabolism in neurons and other cells is the membrane-bound enzyme, Na,K-ATPase. Erythrosin B, Rose Bengal (tetraiodo, tetrabromofluorescein) and diiodofluorescein inhibit Na,K-ATPase in a ouabain-like fashion. In vitro, Erythrosin B and Rose Bengal have IC₅₀ values in the nanomolar range. With incubation times greater than 30 min, their IC₅₀ values are in the picomolar range. Inhibition can be demonstrated in terms of ligand binding to synaptic membranes (3H-ouabain); in terms of ²²Na and ⁸⁶Rb flux into intact synaptosomes; and in terms of catalytic activity of Na-stimulated ATPase in homogenates.

The fluorescein dyes are structurally unlike the cardiac glycosides ouabain and digitalis, yet they appear to be the most potent inhibitors of Na,K-ATPase outside the glycosides. A structure-activity study of 20 fluorescein derivatives demonstrates that halogenation of the dibenzofuran nucleus is required, and that I is more potent than Br as a substituent on the ring. The parent compounds fluorescein and xanthene (or xanthone) are inactive. Some phenolphthalein derivatives may also possess activity against ATPase.

In the course of these studies, we have demonstrated two distinct binding sites for 3H-ouabain in brain tissue. These sites, which are approximately equal in distribution in the brain, have significantly different kinetic properties ($K_d=1$ nM and 100 nM). The artificial dyes appear to interact specifically with the high affinity site, which, on the basis of our and other research, may be an enzyme found only in nerve cell membranes. The dyes are inactive against red blood cell Na,K-ATPase. In addition, there are significant regional differences in the amount of high affinity 3H-ouabain binding in brain, with the lowest amounts in striatum. These complexities in the enzyme may confer highly specific neurobehavioral properties on specific inhibitors such as erythrosin B. In addition, inhibition of Na,K-ATPase can be linked mechanistically to our earlier observations of dye-induced inhibition of sodium dependent uptake, since ouabain can also inhibit the Na-dependent transport processes for reuptake of neurotransmitters by nerve terminals.

Projected Course:

Studies are underway to investigate further the mechanism of nonglycoside inhibition of the enzyme by biophysical, neurochemical, and morphologic measures. In collaboration with the Laboratory of Neurophysiology, NINCDS, we are studying biophysical interactions of erythrosin B with synaptic membranes in order to define sites and nature of binding to the enzyme. We will be using custom-synthesized high specific activity ¹⁴C-erythrosin B in two studies: to localize anatomically the binding of the dye to nerve terminals in vitro by autoradiography, and to determine if the dye can enter the CNS after peripheral administration.

The neurotoxicity of erythrosin B under in vivo conditions simulating human dietary exposure is being studied in a series of experiments to be concluded by September 30, 1980. In these studies, young rats (30 days) are exposed to erythrosin B via their drinking water. Consumption and growth rates were monitored and found not to be changed. Behavior will be studied in these animals after 60 days exposure. Spontaneous motor activity, shuttle box acquisition, and startle reflex will be measured. Neurochemical studies will be done after sacrifice to measure regional Na,K-ATPase in the brain. Based upon the results of these studies, further behavioral and neurochemical studies will be done to determine mechanisms of neurotoxicity and possible age-related susceptibility to exposure.

In addition, because of the unusual biochemical properties of erythrosin B, we are planning experiments to utilize the dye as a reagent to purify brain-specific Na,K-ATPase using affinity column chromatography.

D. Susceptibility to Neurotoxins: Genetic and Environmental Factors:

In order to explore factors which influence response to neurotoxins, we have investigated both genetic and environmental factors. Genetic studies were done by assembling 20 different rat strains available at NIH. Both male and female animals were used for studies of brain receptors, brain enzymes, and hepatic enzymes. For a treatment modelling environmental factors influencing subsequent neurotoxin exposure, we have selected the fetal alcohol syndrome by administering ethanol to pregnant rats.

Major Findings etc.

The following rat strains were studied in detail after a preliminary screen indicated that these strains represented significant differences in brain and hepatic function: ACI/N, Buf/LSC, Buf/N; MNR/N; SD/N. In collaboration with the department of Pharmacology, USUHS, enzymes involved in heme-dependent drug metabolism and heme synthesis in liver and blood were analyzed. Monoamine oxidase (type A and B) were analyzed in liver and brain. Synaptic membrane receptors for dopamine (3H-spiroperidol) and acetylcholine (3H-QNB) were studied in caudate. Activity of brain Na,K-ATPase was studied in cortex by binding of 3H-ouabain and catalysis of ATP. The results indicate significant differences among strains which can be exploited to study differential responses to inhibitors of drug metabolism (such as heavy metals and pesticides), neuroactive agents which affect cholinergic or dopaminergic function (lithium), and inhibitors of Na,K-ATPase (such as the artificial food dyes).

In connection with studies on ethanol as a predisposing factor for neurotoxin susceptibility, we have studied in vitro actions of ethanol on neurotransmitter receptors and Na,K-ATPase. In vivo administration of ethanol has been reported to decrease striatal dopaminergic responses possibly by a direct effect on dopamine receptors. In vitro, however, concentrations of ethanol greater than 35 mM (0.2Δ) inhibit binding of 3H-spiroperidol to synaptic membranes. This inhibition appears to represent a reduced affinity

of the ligand for the receptor. In addition, the decreased receptor affinity produced by ethanol appears to represent effects on both association and dissociation rates for ligand binding. The rates are altered so that the ligand spends less time in association with the receptors. These direct effects of ethanol in vitro indicate that changes in dopamine function, which have been measured behaviorally (and include stereotypy) can be explained by direct effects on receptors. In vivo exposure to ethanol has also been associated with increased activity of Na,K-ATPase in brain and peripheral organs. In vitro, ethanol at concentrations below 0.5M appears to inhibit the ATPase in brain tissue, and, at greater concentrations, to stimulate its activity. These biphasic effects may be important in determining responses to acute and chronic ethanol exposure, as well as dose-related differences in response.

These experiments have provided information on the mechanisms of action of ethanol in the brain. In addition, effects on dopamine receptors and Na,K-ATPase confirm the utility of ethanol exposure as a representative of pre-existing exposures for studying increased susceptibility to heavy metals and food dyes, which we have demonstrated to affect these (and other) neurochemical parameters.

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Developmental Brain Pathology Section, ODIR
National Institute of Neurological and Communicative Disorders and Stroke

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01388-15 ODIR												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Perinatal Asphyxia and its CNS Consequences														
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COOPERATING UNITS (if any) None														
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SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated. The following manuscripts have been published. Mirsky, A.F., Orren, M.M., Stanton, L., Fullerton, B.C., Harris, S., and Myers, R.E.: Auditory evoked potentials and auditory behavior following prenatal and perinatal asphyxia in rhesus monkeys. <i>Develop. Psychobiol.</i> 12: 369-379, 1979. Myers, R.E.: Placental pathology. In Andrews, E.J., Ward, B.C., and Altman, N.H. (Eds.): <u>Spontaneous Animal Models of Human Disease</u> . New York, Academic Press, 1979, pp. 208-209. Myers, R.E.: Maternal anxiety and fetal death. In Zichella, L., and Pancheri, P. (Eds.): <u>PsychoNeuroEndocrinology in Reproduction</u> . Amsterdam, Elsevier/North Holland Biomedical Press, 1979, pp. 555-573.														

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Medical Neurology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report
October 1, 1979 through September 30, 1980
National Institute of Neurological and Communicative Disorders and Stroke
Medical Neurology Branch

John L. Sever, M.D, Acting Chief

Neuromuscular Diseases Section

Introduction: An inter-related multidimensional attack on the chosen target diseases is emphasized in our application of basic research techniques: tissue culture, histochemistry, electronmicroscopy, immunology, autoradiography, biochemistry, and clinical neurophysiology. In the human neurologic disorders studied, these techniques support thrusts to seek: (a) more precise morphologic, electrical, immunologic and chemical definition of the abnormalities; (b) separation of each disorder into more distinct and often new sub-forms; (c) specific or symptomatic treatment; and (d) induced animal models closely related to the human pathophysiologic states. Our emphasis is on the neuromuscular diseases -- they affect more than 1,000,000 persons in the country.

For the clinical investigations, 548 patients were admitted for a total of about 5,840 inpatient days, and there were 2,870 outpatient visits. About 420 patient muscle and nerve biopsies were processed histochemically and reported out -- about 62 of those were from outside hospitals. Many other outside biopsies are submitted for formal opinion. Clinical electrophysiologic studies were performed on 264 patients including 113 consult patients. In the past year 32 articles were published or are in press, and there were more than 50 presentations to meetings.

Approximately 20 neurologists and other physicians and 11 technicians came this past year to learn clinical and laboratory research techniques in neuromuscular diseases. A number of medical students and residents from various other hospitals rotated through our service. Many of our former trainees are full professors, associate professors, and assistant professors in academic departments, and many are directors of Muscular Dystrophy Clinics and Myasthenia Gravis Clinics; many are Medical Advisory Board members of the National Muscular Dystrophy, Myasthenia Gravis, Amyotrophic Lateral Sclerosis, and Multiple Sclerosis associations.

We have international collaborative programs on neuromuscular diseases with investigators in Paris; Sittard, Amsterdam (The Netherlands); San Paulo (Brazil); San Juan (Puerto Rico); Padova, Abeno Terme, Messina, Rome (Italy); Gottingen (Germany); Liege (Belgium); Uppsala (Sweden); Buenos Aires (Argentina); Santiago (Chile); Jerusalem (Israel); Sapporo (Japan).

Myopathies

Myopathies are non-neurogenic, primary or secondary diseases of muscle. Some such as the dermatomyositis/polymyositis group, are often at least partially treatable, but their cause and details of their probably "dysimmune" pathogenesis are not known; others are not treatable but their cause is known, e.g., genetic deficiencies of phosphorylase, phosphofructokinase, acid maltase, carnitine-palmityl-transferase, or carnitine; while still others, such as Duchenne muscular dystrophy and other genetic disorders bearing the name "dystrophy", are of unknown pathogenesis and are untreatable. Some, such as the malignant hyperthermia-rigidity syndrome, are preventable if identified.

A synthetic current personal view of the pathogenesis of a number of neuromuscular diseases has now been published (Dagen Des Oordeels).

I. Inherited Myopathies.

A. Biochemically distinct myopathies.

1. Lysosomal defects. The mechanism of muscle fiber damage is different from that of the afuelias. It probably involves leakage of the excess lysosomal hydrolytic enzymes to dissolve the fiber from within -- an "endo-dissolution". Regeneration is minimal. (a) Acid maltase deficiency (AMD): Previously we have demonstrated a reincarnation of the biochemical and morphologic abnormality in muscle cells cultured from acute-infantile, chronic-infantile and adult-onset forms of the disease, (i) establishing it as a true intrinsic defect of the muscle cell and (ii) providing a new test system for in vitro therapeutic trial, without risk to the patient. This year bio-physical studies of the plasmalemma of the cultured AMD muscle (with NICHD) have shown slightly higher resting membrane potentials (RMP) and lower input resistance than normal early in culture, and as the fibers develop vacuoles the RMP and input resistance fall well below normal. Unlike normal cultured fibers the AMD fibers were early excitable at RMP. This demonstrated a plasmalemmal abnormality intrinsic to the AMD fiber, which possibly results from the excessive acid hydrolases in the AMD fiber.

2. Afuelias: We have introduced the term "afuelias" to describe defects, known and unknown, of (i) glycogen/glucose utilization and (ii) lipid fatty-acid ketone-body utilization. The former cause muscle-fiber breakdown during heavy exercise, especially ischemic exercise -- they include phosphorylase, phosphofructokinase and debrancher-enzyme deficiencies. The latter cause breakdown during fasting states -- they include failure to utilize long-chain fatty-acids, carnitine palmityl transferase deficiency, probably infantile fatal fasting rhabdomyolysis, and now carnitine deficiencies. (a) Glycogen/glucose utilization defects -- these were detailed in last year's report. (b) Lipid/fatty-acid/ketone-body utilization defects. (1) Documented by us previously was the first defect in this category, impaired utilization of long-chain fatty-acids, some further cases of which were found by others to be due to carnitine palmityl transferase (CPT) deficiency. (2) Carnitine deficiency (with VA Hospital, Madison, WI). (a) Although this, surprisingly, does not usually manifest itself as an

afuelia, we have now identified a striking example in muscle-carnitine-deficiency, in a 21-year-old woman suffering repeated rhabdomyolyses in childhood. Alternate fuel pathways were hyperactive: increased plasma ketone bodies, muscle β -OH-butyrate dehydrogenase, and muscle mitochondrial α -glycerophosphate dehydrogenase. She achieved remarkable improvement, from near death and on a respirator (unresponsive to prednisone) to nearly normal with oral carnitine treatment (with U. FL). In contrast to normal muscle, cultures of this patient's muscle failed to grow without addition of 1 mM l-carnitine. (b) A 16-year-old boy with muscle carnitine deficiency, on a respirator and near death, improved to about 75% of normal only after prednisolone was added to high-dose oral carnitine; the facilitatory role of prednisolone in carnitine uptake is being studied in cultured normal and carnitine-deficient human muscle. (c) A baby with muscle carnitine deficiency diagnosed at age 3 mos. is much improved by oral carnitine after 4 mos. of treatment (with NNMCM). (d) l-Carnitine stimulates oxidation of long-chain fatty acids by cultured human skin fibroblasts and muscle; no differences were evident between normals and carnitine deficiency patients (with NHLBI). (e) We confirmed the marked urinary excretion of dicarboxylic acids in muscle carnitine deficiency, presumably caused by increased omega-oxidation of long-chain fatty acids, and now are seeking details of that phenomenon (with NHLBI).

3. Hypocyclasias: Reduced skeletal muscle plasmalemmal adenylate cyclase but normal β -adrenergic receptors we have now reported in three conditions: (a) muscle-fiber-hypotrophy-with-central-nuclei, (b) myotonic atrophy, (c) diazacholesterol-induced myotonia of intact rat and of tissue-cultured rat muscle (last two discussed in Episodic Weakness/Myotonia Project). Now published is our study of muscle cells cultured from affected infants of two families with X-linked recessive infantile-fatal muscle-fiber-hypotrophy-with central nuclei. Both showed the same abnormalities: (1) marked, apparently uncontrolled proliferation, resembling that of neoplastic cells, which persisted through many passages over 10 months, and was not controlled by CNS extract or CNS co-cultures; (2) large multinucleated myotubes formed very early but never matured by light- or electronmicroscopic criteria and never contracted, (3) only 60% of normal adenylate cyclase (basal, and NaF or isoproterenol stimulated) in plasmalemma but normal β -adrenergic receptors (by ^{125}I -hydroxypindolol-binding). These findings demonstrated an intrinsic defect of the muscle cell, apparently an impaired control mechanism(s) related to cAMP. A third patient, has now been treated 6 mos. with phthalazinol, a phosphodiesterase inhibitor, and is improved -- the blinded placebo phase has just begun. If verified, this would be a new treatment developed on the basis of a tissue-culture finding.

4. Other biochemical abnormalities. (a) Ornithine-amino-transferase (OAT) deficiency. This defect is known in gyrate atrophy of retina and choroid, as are the tubular aggregates (of reticulum) of skeletal muscle. In muscle cultured from these patients we demonstrated: (i) OAT deficiency (which is not demonstrable in mature muscle fibers of biopsies), (ii) exquisite intoxication (rapid death) by added ornithine (no effect on control muscle), and (iii) abnormality proliferation of tubules. This tissue-culture system

might be useful in screening putatively therapeutic drugs in this disease.

II. Duchenne muscular dystrophy (DMD). This is the most prevalent of the old-terminology "muscular dystrophies". It is an X-linked hereditary progressive deterioration of muscle in boys, usually causing wheelchair or bed confinement by age 12 and death by age 20 years. Cause and treatment are not known. Current competing hypotheses of the pathogenesis of DMD are: (a) primary or secondary defect of blood supply to muscle, (b) primary defect of energy source within the muscle fiber, and (c) primary muscle-fiber plasmalemmal defect. Although nearly all others favor (c), we favor (a) or (b) or (b + a). Some of the findings in DMD muscle considered by others to be supportive of (c) we consider to be invalid results or not distinguishing between (c) and (b). In DMD the plasmalemma has long been known to be leaky, evidenced by elevated CPK and other "muscle enzymes" in the serum, but that certainly does not specify a primary plasmalemmal defect. Our studies have been concerned with: the nature of the muscle cell plasmalemma, the effects of its leakiness, plasmalemma of other cells in DMD patients, and intramuscular blood vessels and flow.

A. Plasmalemmal Composition. Being reported are our studies with a battery of membrane probes applied to normal and pathologic human and animal muscle cultured aneurally and studied in different developmental states. For example, tannic-acid stained plasmalemma only of mature muscle fibers and thus can be used as a marker of muscle fiber maturity; it also stained t-tubules of rat and chicken muscle cultures and showed these structures absent in cultured human muscle. A new technique of concanavalin A staining of plasmalemma of sections is noted below. Thus, a base established against which cultured Duchenne dystrophy muscle can now be compared.

B. Plasmalemmal porosity: outward leakage. (a) Radioimmunoassay of serum BB-isozyme of creatine kinase (CK) has now been reported as a new method of demonstrating leaking immature or regenerative muscle fibers, while the MM-isozyme demonstrates leaking mature fibers. Although this approach gives an indication whether non-mature or mature fibers are leaking, it has not increased the detection of carriers of Duchenne dystrophy (with CC). (b) Hemopexin is an inducible, liver-produced, heme-transport protein. It was first discovered elevated in DMD patients and carriers a number of years ago by a current member of our group. We have now reported (with UCSD) our confirmation of that. A series of studies in patients and animals, described in last year's annual report, support our hypothesis that the elevation is induced by the subtle myoglobin leakage from damaged muscle fibers. We have also found elevated serum hemopexin in certain active myopathies, especially dermatomyositis/polymyositis and DMD patients and carriers, but also myasthenia gravis patients, all of those groups of patients having had elevated serum myoglobin. Molecular turnover studies, completed this year, using ^{125}I -hemopexin and ^{131}I -albumin showed an increased turnover rate of hemopexin (increased synthesis > increased catabolism) in patients with Duchenne dystrophy, dermatomyositis/polymyositis and myasthenia gravis compared with normal and disease controls. Parallel turnover studies in monkeys showed that small amounts of heme (as could come from myoglobin or intravascular hemolysis) increased hemopexin synthesis while larger amounts of heme

were required to increase the catabolism of hemopexin; those data demonstrated aspects of the hemopexin regulating mechanisms and explain what we observed in patients.

C. Plasmalemmal porosity: inward leakage. A current status report of calcium ingress as part of our "calcium hypothesis" was presented in detail, by request at an international Musclar Dystrophy Association meeting. That ingress is considered the trigger event for muscle responses to impaired plasmalemmal integrity, be it damage of unknown cause as in DMD or of known cause as in an endogenous afuelia or an exogenous ischemia, toxin, toxic-antibody, or toxic T-lymphocyte. If the calcium entry is slight and restricted, it is relatively benign, and probably is the "spark for repair/regeneration/hypertrophy" of muscle fibers. If the calcium entry is severe and unrestricted it begins a lethal cascade of events pushing muscle fibers past their point of no return, i.e., it is probably the "messenger of molecular doom". We have based our calcium hypothesis on our numerous inter-related studies of calcium in normal and damaged muscle fibers of patients with various neuromuscular diseases and various induced animal models thereof, utilizing light- and electronmicroscopic histochemistry, autoradiography, biochemistry and clinical scanning. The calcium mechanism can account for the large amount of obvious and subtle regeneration we see in DMD muscle by acridine orange, alkaline phosphatase, and adenylate cyclase reactions. The calcium mechanism is not disease-specific.

D. Muscle blood vessels and blood flow. Our ischemia hypothesis for DMD, which proposed a functional defect on the arterial side of the vascular tree, was based on our studies of the histochemopathology of DMD muscle, our experimental ischemic myopathy in animals, and our study of human ischemic limb muscles. An ischemia mechanism, although possible in DMD patients, has not yet been demonstrated in them directly. (a) The next logical step we have undertaken has been developing a laser-doppler and radiometric monitor technique for measuring blood flow in superficial capillaries of patients' muscle at time of biopsy and quantitating the vascular responses to perturbation by transient hypoxic stasis (with NHLBI). In initial studies (presented) we have evaluated the technique as applied to human forearm skin blood vessels and to rat skeletal muscle vessels, and their responses, including decrease with occlusion and post-occlusive hyperemia. (b) Our other new techniques for studying muscle blood vessels, now reported, include: (i) autoradiographic localization of β -adrenergic receptor with ^{125}I -hydroxybenzyl-pindolol, showing very high concentration in intramuscular vessels, much higher than in muscle fibers; and (ii) histochemical localization adenylate cyclase, showing it to be much higher in vessels than in normal muscle fibers (but moderately high in regenerative fibers).

E. Collagen increase. Although quite excessive in DMD and suggested by others to have a pathogenic role, it was chemically the same as in polymyositis/dermatomyositis (v.i.).

III. Polymyositis/Dermatomyositis Complex (PM/DM):

PM/DM is an acquired disorder causing progressive deterioration of muscle in

children and adults. The primary cause is not known but the pathogenic mechanism is considered dysimmune (autoimmune). Before the introduction of anti-dysimmune therapy, all patients were seriously incapacitated and many died.

A. Therapeutic efforts: Dysimmune component -- high-single-dose alternate-day prednisone (HSDAD-Pred) seems to be the best treatment. Because of prednisone side-effects and because not all PM/DM patients respond we have been using, successfully, azathioprine 3 mg/kg/day (or cyclophosphamide 2 mg/kg/d). In patients failing to respond to these drugs we have found a new drug Poly-ICLC beneficial in the first few patients treated (see our ALS Project). Total-body-irradiation (TBI), low-dose, because the lymphocytes are the circulating cells most sensitive to TBI, has been dramatically beneficial in one patient with polymyositis who was a failure at multiple drug therapy. On a respirator and about to die -- she now is home and quite strong (with Radiation Therapy, Clinical Center). Calcinosis -- massive subcutaneous calcific-dermatomyositis "calcinosis universalis", is a complication of dermatomyositis which is crippling and causes skin breakdown and infection. Calcium-solubilizing agents (EDTA, diphosphonate) in the past have failed. However, we have found in some severely affected patients the calcium has remarkably diminished as the muscle and skin were responding to our combined azathioprine-HSDAD Pred program, and has remained diminished for several years, even as the drugs were gradually reduced.

B. Pathogenic mechanisms: The exact mechanism(s) of muscle damage in PM/DM is unknown. (1) We previously found immunoglobulin complexes in muscle blood vessels in 83% of children and 29% of adults, supporting the hypothesis of a vascular mechanism of damage, especially in childhood DM. To directly study blood flow in the patients' muscle we will use a laser-doppler and radiometric methods at time of muscle biopsy (see Duchenne dystrophy, above) (with NHLBI). (2) DM/PM is considered to be a dysimmune response to either an exogenous antigen or a normal cell component "foreigned" by an exogenous agent. Specific treatment/elimination of an exogenous agent could be curative (as opposed to the merely suppressive action of all current treatments). We are continuing to seek evidence of an exogenous agent(s). We have not yet been able to rescue an agent nor to find reverse transcriptase. (3) As a possible model for a pathogenic step in PM/DM, cultured normal human muscle has been successfully infected with measles virus; the resultant morphologic abnormalities have been recorded electronmicroscopically and, with our new technique, virus antigen in particles has been localized immunoelectron-microscopically in cytoplasm and nucleoplasm (with NI, NINCDS). (4) Collagen increase in DM/PM has been suggested by others as a possible pathogenic mechanism of muscle fiber damage. We have used antibodies against types I, II, III and IV collagens (which differ by virtue of the amino acids of their 3 polypeptide chains), against their procollagens, and against fibronectin (study in press). We found types I and III collagen and procollagen and fibronectin in the normal endomysium and perimysium; they accumulate there in DM/PM, but in the same manner as in Duchenne dystrophy and thus the accumulation is not disease-specific. These are also increased in intramuscular blood vessels of DM/PM, especially the childhood form, but not in Duchenne dystrophy -- this is more disease-specific but may be

secondary to immunoglobulin complexes deposited in the vessels (v.s.). Type IV collagen normally is only in cellular basement membranes (basal laminae) and is not altered in DM/PM (or DMD).

IV. Other Myopathies and Neuromuscular Diseases of Uncertain Classification:

A. Malignant hyperthermia-rigidity (MHR) is a syndrome, 70% fatal, of acute rise of body temperature accompanied by muscle rigidity during general anesthesia, usually provoked by halothane and/or succinylcholine. A number of the patients (if not all, by definition) have underlying not-well-defined neuromuscular disorders. One well-defined underlying condition is central core disease, having a high incidence of MHR. Not only have we postulated central core disease to be based on an atypical neurogenic mechanism, but we continue to find that when demonstrable muscle pathology is present it is usually ordinary neurogenic atrophy. Thus the possible role of abnormal innervation deserves emphasis in malignant hyperthermia patients.

B. "Ragged-red" muscle fibers, which contain severe mitochondrial abnormalities, heterogenous syndrome of oculocranosomatic neuromuscular diseases with ragged-red fibers (OCSNMD-RR). We have now found similar abnormal mitochondria in other conditions: (i) muscle carnitine deficiency, and (ii) angio-neurotic edema patients who have myotoxic reactions to epsilon-amino-caprylic acid or to androgens (with NIAID).

V. Basic biology of muscle. Several technical advances and new findings have been obtained.

A. Tissue culture. (i) Human muscle biopsies (110 patients) and rat muscle were cultured with improved techniques and used for a variety of cytochemical, immunocytochemical, ultrastructural, biochemical, autoradiographic and electrophysiologic studies (also see other parts of this Project). (ii) New techniques have been developed to allow viable preservation for future cultures of often irreplaceable specimens from patients -- this involves freezing in DMSO of either portions of the original biopsy or early cultures of muscle (or schwann cells) and indefinite storage. The procedure allows valuable human neuromuscular cells to be preserved for future culture when new tests or in vitro therapeutic trials might become available.

B. Electronmicroscopy. A new technique has been developed for the ultra-structural localization of non-specific esterase in normal and abnormal human skeletal muscle. The plasmalemma, outer membrane of mitochondria, T-tubules and tubular aggregates are stained. (ii) A new technique has been developed for the ultrastructural localization of concanavalin-A binding sites in human muscle that had previously been embedded in plastic. This allows us to go back and study many old but valuable biopsies. (iii) A new technique has been developed for ultrastructural immunolocalization of measles virus in plastic-embedded human muscle cultures.

C. Autoradiography. (i) Localization of β -adrenergic receptors in normal and denervated rat muscle with ^{125}I -hydroxybenzylpindolol, now published,

showed in normal muscle much greater amount of β -adrenergic receptors in arterial-tree vessels than in muscle fibers and indicated (a) the fallacy of assuming β -adrenergic binding studied biochemically in whole-tissue muscle homogenates is only in muscle cells, and (b) the potential importance of arterial-tree, as well as muscle fiber, β -adrenergic receptors in human neuromuscular diseases. (ii) To human and animal tissue cryostat sections localization of nicotinic acetylcholine receptors (nAChRs) with ^{125}I - α -bungarotoxin in neuromuscular junctions, in plasmalemma of denervated fibers and in thymic epithelial cells was achieved -- this now makes possible the study of these receptors in routine cryostat sections of all our diagnostic biopsies. Other receptors, e.g., α -adrenergic receptors and insulin receptors will be sought.

D. Biochemistry. Most of our attention has been directed to components of the plasmalemma, t-tubule and reticulum of human and animal muscle, correlated with our other studies of the plasmalemma. Skeletal muscle plasmalemma (PL), sarcoplasmic reticulum (SR), and mitochondria (M) were prepared from homogenates of normal and denervated rat muscle, from human muscle from legs amputated for osteogenic sarcoma, from human diagnostic muscle biopsies, and from patient biopsy muscle grown in tissue culture. (1) β -adrenergic receptor (β AR) adenylate cyclase (AC) system. (a) Animal muscle. (i) Following denervation of rat muscle there is an increase of β -AR's (per mg protein using ^{125}I -hydroxybenzylpindolol binding), not blocked by cyclohexamide, and decrease of AC in PL, SR and M. (ii) An aqueous sciatic nerve factor, as a putative trophic/regulatory factor, applied in vitro at higher concentrations decreased β AR of PL 30-40% in denervated muscle and 10-20% in normal muscle. (iii) Developmentally, in the rat β AR and AC are present from 18-day embryonic states (birth occurs at 21 days) but are not functionally coupled until 3 days after birth; this study was presented. (iv) Rat fast-twitch (FT, EDL) and slow-twitch (ST, soleus) muscles showed differences in their PL AC and β AR activities. In intact muscles catecholamines induced greater production of cAMP in FT than in ST muscle. (b) Human muscle. (i) The subcellular distribution of β AR in normal human muscle was defined and presented. The distribution, PL >> SR > M, correlated with our previous localization of AC in human muscle, supporting a functional coupling. (2) Guanylate cyclase (GC). (a) Animal muscle. (1) We previously reported increase of GC in all fractions (PL, SR, M and 10^5 g supernatant) of denervated rat muscle. We now find the increase is not blocked in cyclohexamide-treated animals, suggesting a mechanism other than new synthesis of GC must be responsible for the increase. (ii) Concanavalin A (Con A) inhibited particulate GC much more in rat denervated muscle than in normal muscle. (b) Human muscle. (i) In normal human muscle the subcellular distribution and properties of GC were similar to those we observed in rat muscle. The specific enzyme activity among subcellular fractions was PL > SR > M > soluble fraction (10^5 g supernatant). Even though PL possessed the highest specific activity, the predominant portion of GC (60%) was in the soluble fraction. The enzyme required Mn^{2+} , but Mg^{2+} could substitute to a considerable extent. Also demonstrated were elution profiles on sepharose columns, substrate dependency, ionic requirements and pH profiles. (ii) Sedimentation profiles of human and rat PL and cytosol GC on 5-20% linear sucrose gradients were determined. PL GC sediments at $S_{20,w}$ of 11.2 while the cytosol gc at 9.1.

(3) Con A Binding greatly increased in denervated rat muscle PL and SR compared to controls. Kinetics of Con A binding PL and SR were determined. (4) Vanadate showed dual effects on PL AC. It activated basal AC but inhibited the activation by NaF and catecholamines.

Amyotrophic Lateral Sclerosis.

I. Amyotrophic Lateral Sclerosis: The disease usually causes death within 2-5 years, although some cases are more chronic. 95% of the cases are sporadic, 5% are dominantly inherited. The cause of ALS is unknown -- dysmetabolic vs. viral are the two main possibilities. We favor the former but are pursuing both.

A. Therapeutic Trials: Four drugs and one procedure studied over the past three years have failed to show general benefit in ALS: they include Phthalazinol (an inhibitor of cAMP phosphodiesterase, and of cGMP phosphodiesterase to a lesser extent) in 25 patients; human leucocyte interferon (an antiviral substance) in one patient; Polyinosinic-polycytidylic-acid-poly-l-lysine (Poly-ICLC) (an inducer of interferon) in three patients (with NIAID); adenine arabinoside (an antiviral compound) in 10 patients, and plasmaphoresis in 3 patients. Phthalazinol has recently caused side-effects of interest: (a) in 4 patients with both diabetes mellitus and ALS, increased glucosemia/glucosuria and diarrhea, and, in one, the possible appearance of a new peptide band in isoelectric-focused CSF; whether the findings can be related is being explored; (b) in all patients as a group, a statistical, slight, reversible, lowering of circulating platelet numbers without chemical evidence of coagulation problems; thus from this "side-effect" of phthalazinol merits consideration for therapeutic trial in the prevention of coronary and cerebrovascular occlusion.

B. CSF biochemical studies: The CSF is a thesaural swamp of interesting central nervous system (CNS) transmitters, peptides and other metabolites. In ALS we have previously found HVA and cAMP to be low, but raising them to normal levels with drug treatment was not associated with clinical benefit. We found cGMP low and have not yet been able to raise it to normal with drug treatment, and so cannot comment on clinical response. A detailed chapter on cyclic nucleotide metabolism and the CSF in ALS and other neuromuscular diseases is about to be published. We are now quantitatively analyzing CSF total fatty acid content and composition by thin-layer and gas chromatography. In ALS patients vs. normals and disease controls, we have worked out the ionic-exchange and liquid chromatographic methods for quantitating bases and nucleosides in patients/ CSF. Enolases, neuron-specific and non-neuronal, by radioimmunoassay in ALS CSF were the same as controls (with NIMH). Cultured motor neurons of 16-day fetal-rat ventral spinal cord serve as test-objects of possibly toxic fluids or agents related to ALS patients. To date, comparing treatment of the cultures for one week with CSF of 9 ALS patients vs. CSF of 10 controls, there was no detrimental effect visible morphologically and the level of neuron-specific enolase (radioimmunoassay) in such treated cultures was not different (with NIMH).

C. Blood biochemical studies: (a) Insulin receptors -- no abnormality of insulin receptors on erythrocytes or leucocytes was detectable (with VA Hospital, Washington, D.C.).

D. Motor Neuron Biology: Histochemical properties of lower motor neurons (LMNs) continue to be explored, seeking special properties of them and disease-characteristic defects thereof. We have applied numerous enzymatic, non-enzymatic and lectin-binding reactions -- to date, and the only ones seeming to be of possible relevance to pathogenesis are the high phosphorylase and low succinate dehydrogenase. Additional reactions, especially ones for peptides and receptors of peptides (and effects of ALS serum and CSF thereon), will be explored over the next year. Bidirectional trophisms between motor neurons, muscle fibers and schwann cells are being studied by isolation of fractions containing putative factors and testing for influences on animal and human motor neurons, schwann cells and muscle fibers in tissue culture.

E. Viral Studies: In ALS patients we have continued to search, in several different ways, for evidence of a viral cause even though this is not our highest suspicion of pathogenesis. To date, all our evidence is negative.

F. Denervated Muscle Some of our ALS patients reported decreased muscle twitchings with amantadine. In surgically denervated rat muscle, levodopa and rimantadine had no effect.

Peripheral Neuropathies:

II. Polyneuropathy (Peripheral Neuropathy): The peripheral neuropathies comprise a group of disorders of various causes, but unknown in more than half the patients. They always cause serious physical handicap sooner or later, sometimes associated with intractable pain and ulceration and, in extreme cases, loss of feet and hands. Our studies seek to delineate the underlying causes and where possible develop a treatment. We also seek fuller understanding of the basic biology and pathologic responses of the lower motor and sensory neurons and their schwann cells. Dysschwannian neuropathies are ones in which the neuronal-axon defect is considered secondary to schwann cell abnormality, whereas in dysneuronal neuropathies the lower motor and/or sensory neuron soma ± axon is the major site of abnormality.

A. Biology of the schwann cell:

(1) Culture of human schwann cells -- To study human dysschwannian neuropathies we have developed techniques to grow reproducibly in tissue-culture human schwann cells obtained from diagnostic nerve biopsies. In primary or co-primary dysschwannian neuropathies (e.g., adrenomyeloneuropathy, metachromatic leucodystrophy, some forms of familial idiopathic "Charcot-Marie-Tooth" neuropathy) the schwann cells in culture should manifest the biochemical defect. That defect, when identified, can be treated in culture, as a screening prior to clinical therapeutic trials. Schwann cells might also express the defect in other neuropathies related to general metabolic defects, such as diabetes mellitus. Our technique enables almost complete elimination

of non-schwann cells. Histochemical, fluorescent and ultrastructural characteristics of normal human schwann cells were determined and have been reported. This now serves as a basis to study various dysschwannian neuropathies. To date, 80 sural nerve biopsies have been cultured and studied with histochemistry and electronmicroscopy. (2) Reproduction of abnormalities in cultured human schwann cells of dysschwannian neuropathies -- (a) In adrenomyeloneuropathy we have detected in the cultured schwann cells ultrastructural and biochemical abnormalities, viz., the presence of very-long-chain fatty acids, indicating a production and amplification of the defect in tissue culture (v.i.). (b) In a patient with a dysschwannian continuous-muscle-fiber-activity syndrome, abnormal acid phosphatase-positive lysosomal inclusions were reproduced in the cultured schwann cells, suggesting the disease is intrinsic in the schwann cells and due to a lysosomal enzyme defect. (3) Cultured rat schwann cells -- For basic biologic studies and for use as a test-object in human diseases (see Immunologic Studies), a technique was established, and presented, for growing in tissue culture sciatic-nerve schwann cells from 3-day old rats. Ultrastructural and histochemical characteristics of the cultured rat schwann cells were identical to those of in vivo studied schwann cells in the very young animal, except for lack of a basement membrane and myelin sheets in the latter.

B. Biochemical Studies: (a) Adrenomyeloneuropathy -- now published are our studies of schwann cells and muscle cells cultured from biopsies of three patients. Ultrastructurally increased amounts of lipid droplets and multilaminar inclusions were present in both cell types. Biochemically increased amounts of very-long-chain-fatty-acids, C₂₂-C₂₆, were present in cultured schwann cells and even to a greater degree in cultured muscle cells. Cultured human skeletal muscle incorporated media-derived fatty-acids into several lipid classes including triglycerides, cholesterol esters, phospholipids and glycolipids. When cultured muscle of the patients was presented with C₂₂:0 or C₂₆:0 free fatty-acids in the medium, it accumulated 4-10 fold greater levels than control cultured muscle. The data demonstrated a generalized metabolic defect, and, for the first time, the defect was identified while the patient was alive. (b) 2,3 cyclic nucleotide phosphohydrolase (CNPase) -- a new assay was developed for this "myelin-specific" enzyme, and CNPase was 2x higher in cultured human schwann cells, even though they were not producing myelin, than in any other cultured control human cells (muscle, fibroblasts). This indicates schwann cells have an increased amount of the enzyme prior to making morphologically detectable amounts of myelin. (b) Substance P -- In CSF it was found by radioimmunoassay to be decreased in our neuropathy patients, but in no other neuromuscular diseases surveyed (with U. Oregon and Harvard). All affected patients had sensory nerve involvement. Decrease of Substance P was only approximately correlated with degree of loss of pain and temperature sensation. This is in press.

C. Immunologic Studies: (1) Criteria for a broader classification of dysimmune peripheral neuropathies have been established. These, and a new approach to the analysis and treatment of dysimmune dysschwannian peripheral neuropathies, have been presented at an NIH Clinical Center Conference. (1) In presumably-dysimmune chronic relapsing neuropathy we have (a) localized

immunoglobulin complexes containing IgG, IgM and C₃ in sural nerves of 10 patients, and suggested the complexes to be a pathogenic mechanism (presented); (b) found unfluctuating monoclonal IgG bands in CSF of chronic relapsing but not the acute "Guillain-Barre" form (which had polyclonal IgG), and suggested the monoclonal band might be a predictor of chronicity (in press) (with IDB); (c) found three cases associated with a "non-secretory" osteosclerotic multiple myeloma with immunoglobulin deposition in peripheral nerve, indicating that to be a likely pathogenic mechanism, and also an associated marked thrombocytosis; and (d) searched for humoral factors using cultured rat schwann cells as the test object.

(3) In presumably dysimmune progressive non-relapsing neuropathy: (a) eleven patients had a circulating monoclonal immunoglobulin spike without detectable myeloma or amyloid and with normal bone marrow -- immunoglobulin light chains were deposited in their biopsied nerves, and we suggested they represented circulating neurotoxic molecules (presented); (b) one patient with IgM-K Waldenstrom's macroglobulinemia and neuropathy had crystal-violet-negative deposits of IgM-K light chains in her sural nerve biopsy and lymphocytes bearing K-chains infiltrating that nerve -- treatment with chlorambucil and prednisone improved her neuropathy and lowered serum IgM levels (presented) (with CC).

(4) In our exploration of the pathogenesis of (occult) plasma-cell dyscrasic form of amyloid neuropathy we have (a) found circulating immunoglobulin light-chains (IgG-kappa > IgG-lambda > IgM lambda) in all 10 pts (presented) (b) postulated non-amyloidogenic Ig light chain fragments as circulating neurotoxic molecules (c) found them deposited in peripheral nerves in 7 of 8 patients examined (which also serves as a new way to diagnose the disease), thus confirming the biochemical studies of Glenner, et. al. (presented), and (d) in a unique case of polyneuropathy, amyloidosis and hypernephroma, crystal-violet-positive amyloid was found in muscle blood vessels and connective tissue but none in nerve; the excised tumor had amyloid of Ig-λ origin biochemically and immunochemically; antibodies against denatured λ-type amyloid protein bound to nerve, muscle and tumor (but antibodies against undenatured λ-protein did not); and by electronmicroscopy deposits typically amyloid were present in nerve (with NIAMDD).

(5) In 3 unrelated cases of hereditary or presumably-hereditary amyloid neuropathy including rapidly and slowly progressive forms, we have identified for the first time by immunocytochemistry that the deposited amyloid is pre-albumin (or has antigenically similarities thereto), confirming the biochemical study of Costa et.al. from Portugal.

(6) In hereditary sensory neuropathy with high serum IgA (which we originally described several years ago), we have now found increased circulating immune complexes (with NCI); anti-dysimmune drug treatment shows initial promising results.

D. Therapy: (a) Polyinosinic-polycytidilic acid poly-l-lysine stabilized with carboxymethyl cellulose (Poly-ICLC) is a new treatment which we have found remarkably successful in patients with chronic, relapsing presumably-dysimmune, dysschwannian polyneuropathy previously unresponsive to prednisone plus azathioprine or cyclophosphamide, and plasmapheresis (with NIAID). One patient has been maintained for 30 months, on Poly-ICLC alone for 12 months. Poly-ICLC raised only trivial levels of measurable interferon in the patients' serum. Although Poly-ICLC is an interferon-inducer, we postulate it may be beneficial in this dysimmune disease by a new action we have found, marked transient lymphocytopenia (down to 10-20% of baseline). Preliminary data show preferential reduction of T-lymphocytes. We have proposed this to be a new antidysimmune treatment potentially beneficial to other dysimmune diseases; we now have also obtained response in previous drug-failure patients with dysimmune dermatomyositis/polymyositis, dysneuronal neuropathy, and one with sub-acute post-infectious demyelinating encephalomyelitis (acute multiple sclerosis) (with Walter Reed and NIAID). (b) Chlorambucil and prednisone in Waldenström's macroglobulinemic peripheral neuropathy, see above (presented). (c) Plasmapheresis was clearly beneficial in one of two patients with chronic dysimmune neuropathy treated for 8 weeks, and now early benefit is evident in three patients with amyloidosis, two with pre-albumin deposits and one with IgG deposits (with NCI). (d) High-single-dose alternate-day prednisone in chronic dysimmune, relapsing or progressive, dysschwannian or dysneuronal, polyneuropathy. Our use has been reported previously. We still find that virtually all our corticosteroid-responsive patients are corticosteroid-dependent, requiring 5-20 mg single-dose q.o.d. to prevent exacerbation. Because of corticosteroid side-effects and because some patients do not respond satisfactorily, we now often use combined treatment, prednisone with 3 mg/kg/ day azathioprine (or 2 mg/kg/day cyclophosphamide), and can achieve better response.

III. Central Nervous System Disorders:

A. Spinocerebellar degenerations comprise disease of various causes, a few known, most not, which always result in serious physical handicap sooner or later in the course of the disease, and sometimes early death and/or mental deterioration. Our studies seek to delineate the underlying causes, where possible attempt to develop a treatment, and define basic cellular pathophysiologic mechanisms. We have found that they virtually always to have a lower-motor-neuron component in the form of a neuropathy, usually dysneuronal infrequently dysschwannian.

B. Progressive spastic paraplegia: This is a progressively crippling syndrome of children and adults. The causes are not known. We have published a newly identified cause, adrenomyeloneuropathy, and have with tissue culture of the patients' muscle and schwann cells demonstrated the biochemical defect for the first time while the patient is living (v.s).

C. Post-infectious demyelinating encephalomyelitis ("acute multiple sclerosis"). One subacute progressing patient improved after treatment with Poly-ICLC (v.i.), suggesting a possible therapeutic role of this drug in this disease, and in the closely related disease multiple sclerosis (with Walter

Reed and NIAID).

Myasthenia Gravis

Myasthenia gravis (MG) is an acquired disorder affecting transmission at the neuromuscular junction, mainly in adults and older children. The primary cause is not known, but the pathogenic mechanism is considered to be dysimmune (or autoimmune). Untreated patients usually are seriously handicapped and many die. Palliative treatment with anticholinesterases and anti-pathogenic treatment, consisting of thymectomy, ACTH and more recently, prednisone, have helped considerably, but much disability, some fatality, and drug side-effects do occur.

A. Therapy.

1. Thymectomy. Last year we discussed our review of 55 consecutive thymectomies done over the past 10 years, which showed that thymectomy is potentially beneficial in all patients with onset in teen-ager or later, and repeat thymectomy can be remarkably beneficial in patients previously improved who subsequently exacerbate and do not respond to medical management. However, not all patients respond satisfactorily, and so other treatments are needed.
2. Prednisone. Long-term high-single-dose alternate-day prednisone (LT-HSDAD-Pred) was introduced to this disease by us 10 years ago, and one child was begun on treatment 14 years ago. Many patients continue to have excellent benefit. However, virtually all responders are, even with very gradual tapering of the LT-HSDAD-Pred, dependent on it, requiring 5-20 mg q.o.d. Prednisone in higher doses reduces circulating lymphocytes ($T > B$), and lymphocyte-response to mitogens ($T\text{-mitogens} > B\text{-mitogens}$), but those effects last no longer than 24-48 hours. Because prednisone has toxicity and because some patients do not respond satisfactorily to it, we are seeking other drugs to be used with prednisone or alone. We have found that azathioprine 3 mg/kg/d added to prednisone seems to provide better control in some MG patients.
3. New prednisone-responsive disorders. To be reported are three patients we have identified who, by detailed investigation, do not have one of the neuromuscular diseases (MG, dysschwannian polyneuropathy, dermatomyositis/polymyositis) known to respond to LT-HSDAD-Pred. Because their severe weakness was out of proportion to minimal or no histochemical and electrophysiologic abnormalities, LT-HSDAD-Pred was tried. Remarkable improvement was achieved in each, with return of weakness each time the dosage was lowered too much. They thus have a new disorder(s).
4. Splenic Radiation. Because lymphocytes are the circulating cells most sensitive to x-irradiation and the spleen harbors a large amount of lymphoid tissue, low-dose splenic radiation was introduced as a treatment of MG patients resistant to all other forms of therapy (with RT, CC). 3 of 5 patients benefited objectively, with no serious side-effects. The benefit was transient (weeks to months).

5. Poly-ICLC. We have found this to be a new anti-dysimmune drug (see ALS and Peripheral Neuropathy project). One MG patient showed apparent improvement on a very short course and one on a longer course showed no response; more patients will be studied.

B. Pathogenesis. Questions regarding pathogenesis include possible altered host (patient) immunologic response, "foreigned" host cells, exogenous agent, role of thymus, role of thymus-produced peptide factors, role of lymphocytes, and role of nicotinic acetylcholine receptor (nAChR).

1. Thymus pathology. The rationale for the empirically-observed benefit of thymectomy (v.s.) is still being sought. In addition to the hyperplastic and thymomatous thymuses, we have now demonstrated that thymuses of older patients considered "atrophic" by pre-existing histopathologic criteria have evident in our fresh-frozen sections many small nests of lymphocytic and epithelial cells that look active -- we have postulated they may have a pathogenic role on the older non-thymomatous MG patient, and so have studied their chemical properties.

2. Thymic epithelial cells. a. Nicotinic acetylcholine receptor (nAChR) -- We have previously demonstrated nAChR in thymic epithelial cells histochemically with peroxidase-labelled α -bungarotoxin, and postulated that the thymic epithelial-cell nAChR may be the molecule foreigned by a putative exogenous agent. b. Thymosin -- This is a thymic hormone capable of repairing, sustaining and facilitating functions of lymphocytes. We have now reported the first cytolocalization of thymosin utilizing antibodies to synthetic thymosin α -₁ -- it is in thymic epithelial cells of normal thymuses and of hyperplastic, "involuting", and thymomatous (i.e. "benign" neoplastic proliferation of epithelial cells) thymuses of MG patients (with George Washington U.). A possible facilitatory role by thymosin produced by the active epithelial cells of MG thymuses in the pathogenesis of MG was postulated. c. Thymic epithelial cell cultures - cultured thymic epithelial cells from normal and MG human thymuses never developed myofibrils; they all had typical desmosomes and tonofibrils, and they formed typical Hassall's

corpuscles. Now we have presented that they (and not fibroblasts) contain thymosin by fluorescent antibody reaction (with George Washington U.). We are currently measuring the amount of thymosin they secrete in culture and will then study regulating mechanisms. We are now seeking evidence of nAChR in the cultured thymic epithelial cells with α -bungarotoxin by electronmicroscopic-cytochemical fluorescence and autoradiographic means.

3. Lymphocytes. a. Thymosin -- with antibody against thymosin α -₁, we have shown thymosin on/in circulating lymphocytes. In MG there is a higher percent of T-lymphocytes positive than normal, and this number is reduced after thymectomy of MG patients. The significance is not known. b. nAChR -- attempts to demonstrate this on thymic T-lymphocytes and circulating lymphocytes of normals, MG patients, and other neuromuscular diseases were negative using autoradiography, scintillation counting, and fluorescence microscopy with α -bungarotoxin labelled in various ways. c. Suppressor cells -- one hypothesis is that MG is caused by a defect of suppressor T-lymphocytes. We

are studying the functional suppressor cells in MG, before and after thymectomy.

4. Hemopexin. We reported last year that serum hemopexin is increased in MG patients, and have now shown that it is due to the much-increased hemopexin synthesis exceeding the slightly-increased hemopexin catabolism. The hemopexin elevation in MG was inexplicable until we found increased myoglobin in the serum of MG patients by use of a very sensitive complement-fixation technique. Together, the findings suggested a small amount of myoglobin leakage may result from a hitherto overlooked minimal subclinical plasmalemmal-leaking myopathy in many MG patients.

Periodic Paralysis

Periodic Paralysis (PP). These are hereditary or acquired disorders causing chronic weakness punctuated by attacks of paralysis. There are potassium-benefited and potassium-provoked forms. Associated metabolic abnormalities are known but the actual pathogenic mechanisms are not. Standard palliative/preventive therapy in the idiopathic hypokalemic form of PP is potassium, more recently acetazolamide, and most recently dichlorphenamide. 1. Treatment. In the hypokalemic form of PP, the treatment we introduced, long-term acetazolamide, has continued to be the best prophylactic agent both for preventing attacks and improving inter-attack weakness. It is now in the textbooks as such. Two of our patients have been treated successfully for more than 14 years. In hypokalemic PP patients not responding to, or worsened by, acetazolamide, we have recently found that dichlorphenamide, another carbonic anhydrase inhibitor, can be dramatically beneficial in improving erstwhile "permanent" weakness; these patients may be a separate subset. Since human muscle contains either no, or very little (per disparate studies of our selves cf. others) carbonic anhydrase, the mechanism of acetazolamide and dichlorphenamide benefit in hypokalemic PP remains unknown. 2. Pathogenesis. Muscle of patients with hypokalemic PP was grown aneurally in tissue culture to obtain fibers free of all neural, circulating and other influences existing in the patient. No abnormalities of growth pattern or light- or electron-microscopic appearance was detectable. Cultured fibers of PP patients will be studied with microelectrodes for plasmalemmal biophysical properties (v.i.).

Myotonic Disorders.

A. Myotonia Congenita and Paramyotonia Congenita. Myotonia is a crippling symptom in these inherited diseases of unknown causes. 1. Clinical Studies. Two years ago we reported acetazolamide as a new treatment providing excellent benefit in patients who had failed to respond to other anti-myotonia agents. It continues to have long-term effectiveness in those and several additional patients. 2. Pathogenesis. Growth in culture of muscle from myotonia congenita patients is normal by light and electronmicroscopy. Biophysical properties of plasmalemma of those cultured fibers is mentioned below.

B. Myotonic Atrophy (Myotonic "Dystrophy"). This is an inherited multi-systemic disease, with progressive muscle weakness and wasting, or unknown

pathogenesis. We have previously raised the possibility of at least a partially neurogenic aspect. With our new concept of "myogenous dys-innervation" we have now extended that hypothesis to include a possible myogenous muscle plasmalemmal non-receptivity to neural short- and long-term trophic influences. (1) Pathogenesis, patient studies. (a) Growth in culture -- muscle fibers cultured from the patients show normal growth and appearance by light- and electron-microscopy. (b) Plasmalemmal biophysical properties of cultured human muscle -- with microelectrodes (with NICHD) we have successfully obtained baseline values in 37 normal fibers: resting membrane potential 51.5 ± 4.6 mV, input resistance 11.6 ± 5.9 M Ω ; only 2 of 34 fibers electrically excitable at RMP, but when hyperpolarized to -80 mV an action potential could be elicited from all with threshold for excitation at 19.3 ± 4.9 mV and action-potential amplitude of $91.7 \pm$ mV, with repetitive trains of action potentials occurring after anodal break. Against these we have now compared 42 fibers cultured from patients with myotonic atrophy and found no difference. A future step will be to study the effect on these parameters of motor neuron innervation in vitro of the cultured human fibers, because, based on these results, we have postulated that in myotonic atrophy an intrinsic muscle-cell abnormality may require motor innervation and/or a circulating factor for full expression. In the cultured myotonic muscle we will also study chloride and other ionic conductances and ionic dysequilibrium challenges. We have also measured in cultured normal human muscle ion channels responsive to applied acetylcholine and values different from rat cultured muscle were found; these will next be studied in cultured human myotonic and periodic paralysis muscle (with LB). (c) Insulin receptors on leucocytes are being studied. (2) Pathogenesis, animal models. Diazacholesterol-induced myotonia in the intact animal we discussed last year, including the lowered adenylate cyclase of muscle. We have now presented a report on the effects of diazacholesterol on cultured rat muscle. The drug caused: change of spontaneous contraction rhythm to more continuous and fibrillation-like movements; electronmicroscopically evident smeared z-discs, disorganized myofibrils, some honey-comb appearance and dilation of sarcoplasmic reticulum, and reduced concanavalin A binding to the plasmalemma; increased mobility of 125 I-Con A biochemically; 40-50% decreased adenylate cyclase (basal, and NaF and isoproterenol stimulated) but normal density and affinity of β -adrenergic receptors, suggesting uncoupling of the latter from adenylate cyclase; and increased guanylate cyclase and cGMP-phosphodiesterase, indicating a greater turnover of cGMP.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01039-18 MN
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PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Amiotrophic Lateral Sclerosis (ALS), Other Lower Motor Neuron Diseases, and Peripheral Neuropathies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Medical Neurology Branch

SECTION

Neuromuscular Diseases

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

7

PROFESSIONAL:

5

OTHER:

2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS

☒ (b) HUMAN TISSUES

☐ (c) NEITHER

☒ (a1) MINDRS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In amyotrophic lateral sclerosis (ALS) and other diseases affecting the lower motor neurons, including peripheral neuropathies and some spinocerebellar degenerations, we are seeking (a) more precise morphologic and chemical definition of the abnormalities; (b) separation of each disorder into more distinct, and often new, subforms; (c) most importantly, specific or symptomatic therapeutic response; (d) new methods of analyzing the abnormalities; and (e) animal models of the human pathophysiologic states.

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(Continued on p. 19)

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Project Description:

Objectives: In amyotrophic lateral sclerosis (ALS) and other diseases affecting the lower motor neurons, including peripheral neuropathies and some spinocerebellar degenerations, we are seeking (a) more precise morphologic and chemical definition of the abnormalities; (b) separation of each disorder into more distinct, and often new, subforms; (c) most importantly, specific or symptomatic therapeutic response; (d) new methods of analyzing the abnormalities; and (e) animal models of the human pathophysiologic states.

Methods Employed: A variety of techniques, encompassing tissue-culture, histochemistry, biochemistry, autoradiography, radionuclide scanning, electrophysiology, electronmicroscopy, and immunology, are applied to patients with the various diseases covered in this category and to induced animal-models. Conducted were therapeutic trials, the efficacy of which was judged by clinical testing, functional evaluation, serial quantitative evaluation of muscle function using an apparatus designed by us for quantitating isometric muscle tension, clinical electrophysiology (including nerve conduction velocities), and biochemical data, especially as reflected in cerebrospinal fluid (CSF).

Major Findings:

I. Amyotrophic Lateral Sclerosis: The disease usually causes death within 2-5 years, although some cases are more chronic. 95% of the cases are sporadic, 5% are dominantly inherited. The cause of ALS is unknown -- dysmetabolic vs. viral are the two main possibilities. We favor the former but are pursuing both.

A. Therapeutic Trials: Four drugs and one procedure studied over the past three years have failed to show general benefit in ALS: they include Phthalazinol (an inhibitor of cAMP phosphodiesterase, and of cGMP phosphodiesterase to a lesser extent) in 25 patients; human leucocyte interferon (an antiviral substance) in one patient; Polyinosinic-polycytidylic-acid-poly-l-lysine (Poly-ICLC) (an inducer of interferon) in three patients (with NIAID); adenine arabinoside (an antiviral compound) in 10 patients, and plasmaphoresis in 3 patients. Phthalazinol has recently caused side-effects of interest: (1) in 4 patients with both diabetes mellitus and ALS, increased glucosemia/glycosuria and diarrhea, and, in one, the possible appearance of a new peptide band in isoelectric-focused CSF; whether the findings can be related is being explored; (2) in all patients as a group, a statistical, slight, reversible, lowering of circulating platelet numbers without clinical evidence of coagulation problems; thus from this "side-effect" of phthalazinol merits consideration for therapeutic trial in the prevention of coronary and cerebrovascular occlusion.

B. CSF biochemical studies: The CSF is a thesaural swamp of interesting central nervous system (CNS) transmitters, peptides and other metabolites. In ALS we have previously found HVA and cAMP to be low, but raising them to

normal levels with drug treatment was not associated with clinical benefit. We found cGMP low and have not yet been able to raise it to normal with drug treatment, and so cannot comment on clinical response. A detailed chapter on cyclic nucleotide metabolism and the CSF in ALS and other neuromuscular diseases is about to be published. We are now quantitatively analyzing CSF total fatty acid content and composition by thin-layer and gas chromatography. In ALS patients vs. normals and disease controls, we have worked out the ionic-exchange and liquid chromatographic methods for quantitating bases and nucleosides in patients; CSF. Enolases, neuron-specific and non-neuronal, by radioimmunoassay in ALS CSF were the same as controls (with NIMH). Cultured motor neurons of 16-day fetal-rat ventral spinal cord serve as test-objects of possibly toxic fluids or agents related to ALS patients. To date, comparing treatment of the cultures for one week with CSF of 9 ALS patients vs. CSF of 10 controls, there was no detrimental effect visible morphologically and the level of neuron-specific enolase (radioimmunoassay) in such treated cultures was not different (with NIMH).

C. Blood biochemical studies: (a) Insulin receptors -- no abnormality of insulin receptors on erythrocytes or leucocytes was detectable (with VA Hospital, Washington, D.C.).

D. Motor Neuron Biology: Histochemical properties of lower motor neurons (LMNs) continue to be explored, seeking special properties of them and disease-characteristic defects thereof. We have applied numerous enzymatic, non-enzymatic and lectin-binding reactions -- to date, and the only ones seeming to be of possible relevance to pathogenesis are the high phosphorylase and low succinate dehydrogenase. Additional reactions, especially ones for peptides and receptors of peptides (and effects of ALS serum and CSF thereon), will be explored over the next year. Bidirectional trophisms between motor neurons, muscle fibers and schwann cells are being studied by isolation of fractions containing putative factors and testing for influences on animal and human motor neurons, schwann cells and muscle fibers in tissue culture.

E. Viral Studies: In ALS patients we have continued to search, in several different ways, for evidence of a viral cause even though this is not our highest suspicion of pathogenesis. To date, all our evidence is negative.

F. Denervated Muscle: Some of our ALS patients reported decreased muscle twitchings with amatadine. In surgically denervated rat muscle, levodopa and amatadine significantly reduced the number of fibrillation potentials, while rimantadine had no effect.

II. Polyneuropathy (Peripheral Neuropathy): The peripheral neuropathies comprise a group of disorders of various causes, but unknown in more than half the patients. They always cause serious physical handicap sooner or later, sometimes associated with intractable pain and ulceration and, in extreme cases, loss of feet and hands. Our studies seek to delineate the underlying causes and where possible develop a treatment. We also seek fuller understanding of the basic biology and pathologic responses of the lower motor and sensory neurons and their schwann cells. Dysschwannian neuropathies are

ones in which the neuronal-axon defect is considered secondary to schwann cell abnormality, whereas in dysneuronal neuropathies the lower motor and/or sensory neuron soma \pm axon is the major site of abnormality.

A. Biology of the schwann cell:

(1) Culture of human schwann cells -- To study human dysschwannian neuropathies we have developed techniques to grow reproducibly in tissue-culture human schwann cells obtained from diagnostic nerve biopsies. In primary or co-primary dysschwannian neuropathies (e.g., adrenomyeloneuropathy, metachromatic leucodystrophy, some forms of familial idiopathic "Charcot-Marie-Tooth" neuropathy) the schwann cells in culture should manifest the biochemical defect. That defect, when identified, can be treated in culture, as a screening prior to clinical therapeutic trials. Schwann cells might also express the defect in other neuropathies related to general metabolic defects, such as diabetes mellitus. Our technique enables almost complete elimination of non-schwann cells. Histochemical, fluorescent and ultrastructural characteristics of normal human schwann cells were determined and have been reported. This now serves as a basis to study various dysschwannian neuropathies. To date, 80 sural nerve biopsies have been cultured and studied with histochemistry and electronmicroscopy. (2) Reproduction of abnormalities in cultured human schwann cells of dysschwannian neuropathies -- (a) In adrenomyeloneuropathy we have detected in the cultured schwann cells ultrastructural and biochemical abnormalities, viz., the presence of very-long-chain fatty acids, indicating a production and amplification of the defect in tissue culture (v.i.). (b) In a patient with a dysschwannian continuous-muscle-fiber-activity syndrome, abnormal acid phosphatase-positive lysosomal inclusions were reproduced in the cultured schwann cells, suggesting the disease is intrinsic in the schwann cells and due to a lysosomal enzyme defect. (3) Cultured rat schwann cells -- For basic biologic studies and for use as a test-object in human diseases (see Immunologic Studies), a technique was established, and presented, for growing in tissue culture sciatic-nerve schwann cells from 3-day old rats. Ultrastructural and histochemical characteristics of the cultured rat schwann cells were identical to those of in vivo studied schwann cells in the very young animal, except for lack of a basement membrane and myelin sheets in the latter.

B. Biochemical Studies: (a) Adrenomyeloneuropathy -- now published are our studies of schwann cells and muscle cells cultured from biopsies of three patients. Ultrastructurally increased amounts of lipid droplets and multilaminar inclusions were present in both cell types. Biochemically increased amounts of very-long-chain fatty-acids, C₂₂-C₂₆, were present in cultured schwann cells and even to a greater degree in cultured muscle cells. Cultured human skeletal muscle incorporated media-derived fatty-acids into several lipid classes including triglycerides, cholesterol esters, phospholipids and glycolipids. When cultured muscle of the patients was presented with C_{22:0} or C_{26:0} free fatty-acids in the medium, it accumulated 4-10 fold greater levels than control cultured muscle. The data demonstrated a generalized metabolic defect, and, for the first time, the defect was identified

while the patient was alive. (b) 2,3 cyclic nucleotide phosphohydrolase (CNPase) -- a new assay was developed for this "myelin-specific" enzyme, and CNPase was 2x higher in cultured human schwann cells, even though they were not producing myelin, than in any other cultured control human cells (muscle, fibroblasts). This indicates schwann cells have an increased amount of the enzyme prior to making morphologically detectable amounts of myelin. (b) Substance P -- In CSF it was found by radioimmunoassay to be decreased in our neuropathy patients, but in no other neuromuscular diseases surveyed (with U. Oregon and Harvard). All affected patients had sensory nerve involvement. Decrease of Substance P was only approximately correlated with degree of loss of pain and temperature sensation. This is in press.

C. Immunologic Studies: (1) Criteria for a broader classification of dysimmune peripheral neuropathies have been established. These, and a new approach to the analysis and treatment of dysimmune dysschwannian peripheral neuropathies, have been presented at an NIH Clinical Center Conference. (2) In presumably-dysimmune chronic relapsing neuropathy we have (a) localized immunoglobulin complexes containing IgG, IgM and C₃ in sural nerves of 10 patients, and suggested the complexes to be a pathogenic mechanism (presented); (b) found unfluctuating monoclonal IgG bands in CSF of chronic relapsing but not the acute "Guillain-Barre" form (which had polyclonal IgG), and suggested the monoclonal band might be a predictor of chronicity (in press) (with IDB); (c) found three cases associated with a "non-secretory" osteosclerotic multiple myeloma with immunoglobulin deposition in peripheral nerve, indicating that to be a likely pathogenic mechanism, and also an associated marked thrombocytosis; and (d) searched for humoral factors using cultured rat schwann cells as the test object.

(3) In presumably dysimmune progressive non-relapsing neuropathy: (a) eleven patients had a circulating monoclonal immunoglobulin spike without detectable myeloma or amyloid and with normal bone marrow -- immunoglobulin light chains were deposited in their biopsied nerves, and we suggested they represented circulating neurotoxic molecules (presented); (b) one patient with IgM-K Waldenstrom's macroglobulinemia and neuropathy had crystal-violet-negative deposits of IgM-K light chains in her sural nerve biopsy and lymphocytes bearing K-chains infiltrating that nerve -- treatment with chlorambucil and prednisone improved her neuropathy and lowered serum IgM levels (presented) (with CC).

(4) In our exploration of the pathogenesis of (occult) plasma-cell dyscrasic form of amyloid neuropathy we have (a) found circulating immunoglobulin light-chains (IgG-kappa > IgG-lambda > IgM lambda) in all 10 pts (presented); (b) postulated non-amyloidogenic Ig light chain fragments as circulating neurotoxic molecules; (c) found them deposited in peripheral nerves in 7 of 8 patients examined (which also serves as a new way to diagnose the disease), thus confirming the biochemical studies of Glenner, et. al., (presented); and (d) in a unique case of polyneuropathy, amyloidosis and hypernephroma, crystal-violet-positive amyloid was found in muscle blood vessels and connective tissue but none in nerve; the excised tumor had amyloid of Ig-λ origin biochemically and immunochemically; antibodies against denatured

λ -type amyloid protein bound to nerve, muscle and tumor (but antibodies against undenatured λ -protein did not); and by electronmicroscopy deposits typically amyloid were present in nerve (with NIAMDD).

(5) In 3 unrelated cases of hereditary or presumably-hereditary amyloid neuropathy including rapidly and slowly progressive forms, we have identified for the first time by immunocytochemistry that the deposited amyloid is pre-albumin (or has antigenically similarities thereto), confirming the biochemical study of Costa, et.al., from Portugal.

(6) In hereditary sensory neuropathy with high serum IgA (which we originally described several years ago), we have now found increased circulating immune complexes (with NCI); anti-dysimmune drug treatment shows initial promising results.

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A. Spinocerebellar degenerations comprise disease of various causes, a few known, most not, which always result in serious physical handicap sooner or later in the course of the disease, and sometimes early death and/or mental deterioration. Our studies seek to delineate the underlying causes, where possible attempt to develop a treatment, and define basic cellular patho-physiologic mechanisms. We have found that they virtually always have a lower-motor-neuron component in the form of a neuropathy, usually dys-neuronal, infrequently dysschwannian.

B. Progressive spastic paraplegia: This is a progressively crippling syndrome of children and adults. The causes are not known. We have published a newly identified cause, adrenomyeloneuropathy, and have with tissue culture of the patients' muscle and schwann cells demonstrated the biochemical defect for the first time while the patient is living (v.s).

C. Post-infectious demyelinating encephalomyelitis ("acute multiple sclerosis"). One subacute progressing patient improved after treatment with Poly-ICLC (v.i.), suggesting a possible therapeutic role of this drug in this disease, and in the closely related disease multiple sclerosis (with Walter Reed and NIAID).

Significance to Bio-Medical Research and the Program of the Institute: These findings provide new information (a) on the pathologic and pathogenic aspects of the various lower motor neuron disorders, peripheral neuropathies, spinocerebellar degenerations, and progressive spastic paraplegias, (b) on the treatment of some, and (c) on animal-models of some of these disorders.

Proposed course of Project: To more fully develop the interlinked basic and clinical studies, underway, directed toward clarification of the pathogenesis and identification of the etiology, and, most importantly, toward elaboration of means of treatment and prevention of these disorders.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01034-18 MN
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) Myopathies		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PIs: W. King Engel, M.D., Chief, NMD, MNB, NINCDS Valerie Askanas, M.D., Ph.D., Associate Neurologist, NMD, MNB, NINCDS N. B. Reddy, Ph.D., MNB, NINCDS OTHERS: B. Lavenstein, M.D., MNB, NINCDS A. Tahmoush, M.D., MNB, NINCDS M. Foidart, M.D., MNB, NINCDS M. Dalakas, M.D., MNB, NINCDS B. Joshi, M.D., MNB, NINCDS I. Yaar, M.D., MNB, NINCDS B. Blumenkopf, M.D., MNB, NINCDS A. Galdi, M.D., MNB, NINCDS M. Zweig, M.D., CP, CC A. Lichter, M.D., RO, NCI (Continued below)		
COOPERATING UNITS (if any) CP, CC, NIH; NEI; NICHD; NHLBI; Institut de Pathologie Moleculaire, Paris; NIAID; NMNC; UCSD; VA Hosp., Boston, MA and Madison, WI; IDB, NINCDS; Johns Hopkins University, MD; Palo Alto Medical Clinic, CA; Downstate Medical Center, NY; Univ. of Florida		
LAB/BRANCH Medical Neurology Branch		
SECTION <p style="text-align: center;">Neuromuscular Diseases Section</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, MD 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">6.5</p>	PROFESSIONAL: <p style="text-align: center;">4.5</p>	OTHER: <p style="text-align: center;">2.0</p>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) To more fully elaborate the clinical, tissue-cultural, histochemical, biochemical, ultrastructural, radioisotopic electrophysiologic and immunologic abnormalities of patients with the various <u>myopathies</u> and certain other neuromuscular disorders. To further sub-classify patients in each category using those parameters. To seek pathogenic mechanisms, using a variety of different techniques including ones listed above, applied to the patient's body fluids and tissues, particularly to the <u>muscle biopsy</u> specimens. To tissue-culture human abnormal muscle in order to reincarnate the disease in culture and then to treat it <u>in vitro</u> . To induce, by chemicals and by immunologic means, models of human myopathies in animals and in tissue-cultured human and animal muscle.		
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Objectives: To more fully elaborate the clinical, tissue-cultural, histochemical, biochemical, ultrastructural, radioisotopic electrophysiologic and immunologic abnormalities of patients with the various myopathies and certain other neuromuscular disorders. To further subclassify patients in each category using those parameters. To seek pathogenic mechanisms, using a variety of different techniques including ones listed above, applied to patients' body fluids and tissues, and especially to the muscle biopsy specimens. To tissue-culture human abnormal muscle in order to reincarnate the disease in culture and then to treat it in vitro. To induce, with chemicals, or by immunologic means, models of human myopathies in animals, and in tissue-cultured human and animal muscle. Especially, to treat myopathic disorders by different methods in order to learn which is most effective within each disease category.

Methods Employed: A variety of techniques encompassing tissue-culture, histochemistry, biochemistry, autoradiography, radionuclide scanning, electrophysiology, electronmicroscopy, and immunology are applied to patients with the various myopathies their tissue-cultured muscle, and induced animal-models thereof.

Patient Material: Patients and diagnostic material from Medical Neurology Branch patients and from outside patients from whom diagnostic muscle biopsies were obtained and sent here for study.

Major Findings:

Myopathies are non-neurogenic, primary or secondary diseases of muscle. Some such as the dermatomyositis/polymyositis group, are often at least partially treatable, but their cause and details of their probably "dysimmune" pathogenesis are not known; others are not treatable but their cause is known, e.g., genetic deficiencies of phosphorylase, phosphofructokinase, acid maltase, carnitine-palmityl-transferase, or carnitine; while still others, such as Duchenne muscular dystrophy and other genetic disorders bearing the name "dystrophy", are of unknown pathogenesis and are untreatable. Some, such as the malignant hyperthermia-rigidity syndrome, are preventable if identified.

A synthetic current personal view of the pathogenesis of a number of neuromuscular diseases has not been published (Dagen Des Oordeels).

I. Inherited Myopathies.

A. Biochemically distinct myopathies.

1. Lysosomal defects. The mechanism of muscle fiber damage is different from that of the afuelias. It probably involves leakage of the excess lysosomal hydrolytic enzymes to dissolve the fiber from within -- an "endo-dissolution". Regeneration is minimal. (a) Acid maltase deficiency (AMD): Previously we have demonstrated a reincarnation of the biochemical and morphologic abnormality in muscle cells cultured from acute-infantile, chronic-

infantile and adult-onset forms of the disease, (i) establishing it as a true intrinsic defect of the muscle cell and (ii) providing a new test system for in vitro therapeutic trial, without risk to the patient. This year bio-physical studies of the plasmalemma of the cultured AMD muscle (with NICHD) have shown slightly higher resting membrane potentials (RMP) and lower input resistance than normal early in culture, and as the fibers develop vacuoles the RMP and input resistance fall well below normal. Unlike normal cultured fibers the AMD fibers early excitable at RMP. This demonstrated a plasma-lemmal abnormality intrinsic to the AMD fiber, which possibly results from the excessive acid hydrolases in the AMD fiber.

2. Afuelias: We have introduced the term "afuelias" to describe defects, known and unknown, of (i) glycogen/glucose utilization and (ii) lipid fatty-acid ketone-body utilization. The former cause muscle-fiber breakdown during heavy exercise, especially ischemic exercise -- they include phosphorylase, phosphofructokinase and debrancher-enzyme deficiencies. The latter cause breakdown during fasting states -- they include failure to utilize long-chain fatty-acids, carnitine palmityl transferase deficiency, probably infantile fatal fasting rhabdomyolysis, and now carnitine deficiencies. (a) Glycogen/glucose utilization defects -- these were detailed in last year's report. (b) Lipid/fatty-acid/ketone-body utilization defects. (1) Documented by us previously was the first defect in this category, impaired utilization of long-chain fatty-acids, some further cases of which were found by others to be due to carnitine palmityl transferase (CPT) deficiency. (2) Carnitine deficiency (with VA Hospital, Madison, WI). (a) Although this, surprisingly, does not usually manifest itself as an afuelia, we have now identified a striking example in muscle-carnitine-deficiency, in a 21-year-old woman suffering repeated rhabdomyolyses in childhood. Alternate fuel pathways were hyperactive: increased plasma ketone bodies, muscle β -OH-butyrate dehydrogenase, and muscle mitochondrial α -glycerophosphate dehydrogenase. She achieved remarkable improvement, from near death and on a respirator (unresponsive to prednisone) to nearly normal with oral carnitine treatment (with U. FL). In contrast to normal muscle, cultures of this patient's muscle failed to grow without addition of 1 mM l-carnitine. (b) A 16-year-old boy with muscle carnitine deficiency, on a respirator and near death, improved to about 75% of normal only after prednisolone was added to high-dose oral carnitine; the facilitatory role of prednisolone in carnitine uptake is being studied in cultured normal and carnitine-deficient human muscle. (c) A baby with muscle carnitine deficiency diagnosed at age 3 mos. is much improved by oral carnitine after 4 mos. of treatment (with NNC). (d) l-Carnitine stimulates oxidation of long-chain fatty acids by cultured human skin fibroblasts and muscle; no differences were evident between normals and carnitine deficiency patients (with NHLBI). (e) We confirmed the marked urinary excretion of dicarboxylic acids in muscle carnitine deficiency, presumably caused by increased omega-oxidation of long-chain fatty acids, and now are seeking details of that phenomenon (with NHLBI).

3. Hypocyclasias: Reduced skeletal muscle plasmalemmal adenylate cyclase but normal β -adrenergic receptors we have now reported in three con-

ditions: (a) muscle-fiber-hypotrophy-with-central-nuclei, (b) myotonic atrophy, (c) diazacholesterol-induced myotonia of intact rat and of tissue-cultured rat muscle (last two discussed in Episodic Weakness/Myotonia Project). Now published is our study of muscle cells cultured from affected infants of two families with X-linked recessive infantile-fatal muscle-fiber-hypotrophy with central nuclei. Both showed the same abnormalities: (1) marked, apparently uncontrolled proliferation, resembling that of neoplastic cells, which persisted through many passages over 10 months, and was not controlled by CNS extract or CNS co-cultures; (2) large multinucleated myotubes formed very early but never matured by light- or electronmicroscopic criteria and never contracted, (3) only 60% of normal adenylate cyclase (basal, and NaF or isoproterenol stimulated) in plasmalemma but normal β -adrenergic receptors (by ^{125}I -hydroxypindolol-binding). These findings demonstrated an intrinsic defect of the muscle cell, apparently an impaired control mechanism(s) related to cAMP. A third patient, has now been treated 6 mos. with phthalazinol, a phosphodiesterase inhibitor, and is improved -- the blinded placebo phase has just begun. If verified, this would be a new treatment developed on the basis of a tissue-culture finding.

4. Other biochemical abnormalities. (a) Ornithine-amino-transferase (OAT) deficiency. This defect is known in gyrate atrophy of retina and choroid, as are the tubular aggregates (of reticulum) of skeletal muscle. In muscle cultured from these patients we demonstrated: (i) OAT deficiency (which is not demonstrable in mature muscle fibers of biopsies), (ii) exquisite intoxication (rapid death) by added ornithine (no effect on control muscle), and (iii) abnormality proliferation of tubules. This tissue-culture system might be useful in screening putatively therapeutic drugs in this disease.

II. Duchenne muscular dystrophy (DMD). This is the most prevalent of the old-terminology "muscular dystrophies". It is an X-linked hereditary progressive deterioration of muscle in boys, usually causing wheelchair or bed confinement by age 12 and death by age 20 years. Cause and treatment are not known. Current competing hypotheses of the pathogenesis of DMD are: (a) primary or secondary defect of blood supply to muscle, (b) primary defect of energy source within the muscle fiber, and (c) primary muscle-fiber plasmalemmal defect. Although nearly all others favor (c), we favor (a) or (b) or (b + a). Some of the findings in DMD muscle considered by others to be supportive of (c) we consider to be invalid results or not distinguishing between (c) and (b). In DMD the plasmalemma has long been known to be leaky, evidenced by elevated CPK and other "muscle enzymes" in the serum, but that certainly does not specify a primary plasmalemmal defect. Our studies have been concerned with: the nature of the muscle cell plasmalemma, the effects of its leakiness, plasmalemma of other cells in DMD patients, and intramuscular blood vessels and flow.

A. Plasmalemmal Composition. Being reported are our studies with a battery of membrane probes applied to normal and pathologic human and animal muscle cultured aneurally and studied in different developmental states. For example, tannic-acid stained plasmalemma only of mature muscle fibers and thus can be used as a marker of muscle fiber maturity; it also stained t-

tubules of rat and chicken muscle cultures and showed these structures absent in cultured human muscle. A new technique of concanavalin A staining of plasmalemma of sections is noted below. Thus, a base established against which cultured Duchenne dystrophy muscle can now be compared.

B. Plasmalemmal porosity: outward leakage. (a) Radioimmunoassay of serum BB-isozyme of creatine kinase (CK) has now been reported as a new method of demonstrating leaking immature or regenerative muscle fibers, while the MM-isozyme demonstrates leaking mature fibers. Although this approach gives an indication whether non-mature or mature fibers are leaking, it has not increased the detection of carriers of Duchenne dystrophy (with CC). (b) Hemopexin is an inducible, liver-produced, heme-transport protein. It was first discovered elevated in DMD patients and carriers a number of years ago by a current member of our group. We have now reported (with UCSD) our confirmation of that. A series of studies in patients and animals, described in last year's annual report, support our hypothesis that the elevation is induced by the subtle myoglobin leakage from damaged muscle fibers. We have also found elevated serum hemopexin in certain active myopathies, especially dermatomyositis/polymyositis and DMD patients and carriers, but also myasthenia gravis patients, all of those groups of patients having had elevated serum myoglobin. Molecular turnover studies, completed this year, using ^{125}I -hemopexin and ^{131}I -albumin showed an increased turnover rate of hemopexin (increased synthesis > increased catabolism) in patients with Duchenne dystrophy, dermatomyositis/polymyositis and myasthenia gravis compared with normal and disease controls. Parallel turnover studies in monkeys showed that small amounts of heme (as could come from myoglobin or intravascular hemolysis) increased hemopexin synthesis while larger amounts of heme were required to increase the catabolism of hemopexin; those data demonstrated aspects of the hemopexin regulating mechanisms and explain what we observed in patients.

C. Plasmalemmal porosity: inward leakage. (a) A current status report of calcium ingress as part of our "calcium hypothesis" was presented in detail, by request at an international Muscular Dystrophy Association meeting. That ingress is considered the trigger event for muscle responses to impaired plasmalemmal integrity, be it damage of unknown cause as in DMD or of known cause as in an endogenous afuelia or an exogenous ischemia, toxin, toxic-antibody, or toxic T-lymphocyte. If the calcium entry is slight and restricted, it is relatively benign, and probably is the "spark for repair/regeneration/hypertrophy" of muscle fibers. If the calcium entry is severe and unrestricted it begins a lethal cascade of events pushing muscle fibers past their point of no return, i.e., it is probably the "messenger of molecular doom". We have based our calcium hypothesis on our numerous inter-related studies of calcium in normal and damaged muscle fibers of patients with various neuromuscular diseases and various induced animal models thereof, utilizing light- and electronmicroscopic histochemistry, autoradiography, biochemistry and clinical scanning. The calcium mechanism can account for the large amount of obvious and subtle regeneration we see in DMD muscle by acridine orange, alkaline phosphatase, and adenylate cyclase reactions. The calcium mechanism is not disease-specific.

D. Muscle blood vessels and blood flow. Our ischemia hypothesis for DMD, which proposed a functional defect on the arterial side of the vascular tree, was based on our studies of the histochemopathology of DMD muscle, our experimental ischemic myopathy in animals, and our study of human ischemic limb muscles. An ischemia mechanism, although possible in DMD patients, has not yet been demonstrated in them directly. (a) The next logical step we have undertaken has been developing a laser-doppler and radiometric monitor technique for measuring blood flow in superficial capillaries of patients' muscle at time of biopsy and quantitating the vascular responses to perturbation by transient hypoxic stasis (with NHLBI). In initial studies (presented) we have evaluated the technique as applied to human forearm skin blood vessels and to rat skeletal muscle vessels, and their responses, including decrease with occlusion and post-occlusive hyperemia. (b) Our other new techniques for studying muscle blood vessels, now reported, include: (i) autoradiographic localization of β -adrenergic receptor with ^{125}I -hydroxybenzyl-pindolol, showing very high concentration in intramuscular vessels, much higher than in muscle fibers; and (ii) histochemical localization adenylate cyclase, showing it to be much higher in vessels than in normal muscle fibers (but moderately high in regenerative fibers).

E. Collagen increase. Although quite excessive in DMD and suggested by others to have a pathogenic role, it was chemically the same as in polymyositis/dermatomyositis (v.i.).

III. Polymyositis/Dermatomyositis Complex (PM/DM):

PM/DM is an acquired disorder causing progressive deterioration of muscle in children and adults. The primary cause is not known but the pathogenic mechanism is considered dysimmune (autoimmune). Before the introduction of anti-dysimmune therapy, all patients were seriously incapacitated and many died.

A. Therapeutic efforts: Dysimmune component -- high-single-dose-alternate-day prednisone (HSDAD-Pred) seems to be the best treatment. Because of prednisone side-effects and because not all PM/DM patients respond we have been using, successfully, azathioprine 3 mg/kg/day (or cyclophosphamide 2 mg/kg/d). In patients failing to respond to these drugs we have found a new drug Poly-ICLC beneficial in the first few patients treated (see our ALS Project). Total-body-irradiation (TBI), low dose, because the lymphocytes are the circulating cells most sensitive to TBI, has been dramatically beneficial in one patient with polymyositis who was a failure at multiple drug therapy. On a respirator and about to die -- she now is home and quite strong (with RT, CC). Calcinosis -- massive subcutaneous calcification, "calcinosis universalis", is a complication of dermatomyositis which is crippling and causes skin breakdown and infection. Calcium-solubilizing agents (EDTA, diphosphonate) in the past have failed. However, we have found in some severely affected patients the calcium has remarkably diminished as the muscle and skin were responding to our combined azathioprine-HSDAD Pred program, and has remained diminished for several years, even as the drugs were gradually reduced.

B. Pathogenic mechanisms: The exact mechanism(s) of muscle damage in PM/DM is unknown. (1) We previously found immunoglobulin complexes in muscle blood vessels in 83% of children and 29% of adults, supporting the hypothesis of a vascular mechanism of damage, especially in childhood DM. To directly study blood flow in the patients' muscle we will use a laser-doppler and radiometric methods at time of muscle biopsy (see Duchenne dystrophy, above) (with NHLBI). (2) DM/PM is considered to be a dysimmune response to either an exogenous antigen or a normal cell component "foreigned" by an exogenous agent. Specific treatment/elimination of an exogenous agent could be curative (as opposed to the merely suppressive action of all current treatments). We are continuing to seek evidence of an exogenous agent(s). We have not yet been able to rescue an agent nor to find reverse transcriptase. (3) As a possible model for a pathogenic step in PM/DM, cultured normal human muscle has been successfully infected with measles virus; the resultant morphologic abnormalities have been recorded electronmicroscopically and, with our new technique, virus antigen in particles has been localized immunoelectron-microscopically in cytoplasm and nucleoplasm (with NI, NINCDS). (4) Collagen increase in DM/PM has been suggested by others as a possible pathogenic mechanism of muscle fiber damage. We have used antibodies against types I, II, III and IV collagens (which differ by virtue of the amino acids of their 3 polypeptide chains), against their procollagens, and against fibronectin (study in press). We found types I and III collagen and procollagen and fibronectin in the normal endomysium and perimysium; they accumulate there in DM/PM, but in the same manner as in Duchenne dystrophy and thus the accumulation is not disease-specific. These are also increased in intramuscular blood vessels of DM/PM, especially the childhood form, but not in Duchenne dystrophy -- this is more disease-specific but may be secondary to immunoglobulin complexes deposited in the vessels (v.s.). Type IV collagen normally is only in cellular basement membranes (basal laminae) and is not altered in DM/PM (or DMD).

IV. Other Myopathies and Neuromuscular Diseases of Uncertain Classification:

A. Malignant hyperthermia-rigidity (MHR) is a syndrome, 70% fatal, of acute rise of body temperature accompanied by muscle rigidity during general anesthesia, usually provoked by halothane and/or succinylcholine. A number of the patients (if not all, by definition) have underlying not-well-defined neuromuscular disorders. One well-defined underlying condition is central core disease, having a high incidence of MHR. Not only have we postulated central core disease to be based on an atypical neurogenic mechanism, but we continue to find that when demonstrable muscle pathology is present it is usually ordinary neurogenic atrophy. Thus the possible role of abnormal innervation deserves emphasis in malignant hyperthermia patients.

B. "Ragged-red" muscle fibers, which contain severe mitochondrial abnormalities, are the commonest histochemical manifestation in limb muscles of the heterogenous syndrome of oculocraniosomatic neuromuscular diseases with ragged-red fibers (OCSNMD-RR). We have now found similar abnormal mitochondria in other conditions: (i) muscle carnitine deficiency, and (ii) angio-

neurotic edema patients who have myotoxic reactions to epsilon-amino-caprylic-acid or to androgens (with NIAID).

V. Basic biology of muscle. Several technical advances and new findings have been obtained.

A. Tissue culture. (i) Human muscle biopsies (110 patients) and rat muscle were cultured with improved techniques and used for a variety of cytochemical, immunocytochemical, ultrastructural, biochemical, autoradiographic and electrophysiologic studies (also see other parts of this Project). (ii) New techniques have been developed to allow viable preservation for future cultures of often irreplaceable specimens from patients -- this involves freezing in DMSO of either portions of the original biopsy or early cultures of muscle (or schwann cells) and indefinite storage. The procedure allows valuable human neuromuscular cells to be preserved for future culture when new tests or in vitro therapeutic trials might become available.

B. Electronmicroscopy. A new technique has been developed for the ultra-structural localization of non-specific esterase in normal and abnormal human skeletal muscle. The plasmalemma, outer membrane of mitochondria, T-tubules and tubular aggregates are stained. (i) A new technique has been developed for the ultrastructural localization of concanavalin-A binding sites in human muscle that had previously been embedded in plastic. This allows us to go back and study many old but valuable biopsies. (ii) A new technique has been developed for ultrastructural immunolocalization of measles virus in plastic-embedded human muscle cultures.

C. Autoradiography. (i) Localization of β -adrenergic receptors in normal and denervated rat muscle with ^{125}I -hydroxybenzylpindolol, now published, showed in normal muscle much greater amount of β -adrenergic receptors in arterial-tree vessels than in muscle fibers and indicated (a) the fallacy of assuming β -adrenergic binding studied biochemically in whole-tissue muscle homogenates is only in muscle cells, and (b) the potential importance of arterial-tree, as well as muscle fiber, β -adrenergic receptors in human neuromuscular diseases. (ii) To human and animal tissue cryostat sections localization of nicotinic acetylcholine receptors (nAChRs) with ^{125}I - α -bungarotoxin in neuromuscular junctions, in plasmalemma of denervated fibers and in thymic epithelial cells was achieved -- this now makes possible the study of these receptors in routine cryostat sections of all our diagnostic biopsies. Other receptors, e.g., α -adrenergic receptors and insulin receptors will be sought.

E. Biochemistry. Most of our attention has been directed to components of the plasmalemma, t-tubule and reticulum of human and animal muscle, correlated with our other studies of the plasmalemma. Skeletal muscle plasmalemma (PL), sarcoplasmic reticulum (SR), and mitochondria (M) were prepared from homogenates of normal and denervated rat muscle, from human muscle from legs amputated for osteogenic sarcoma, from human diagnostic muscle biopsies, and from patient biopsy muscle grown in tissue culture. (1) β -adrenergic receptor (β AR) adenylate cyclase (AC) system. (a) Animal muscle. (i) Following denervation of rat muscle there is an increase of β AR's (per mg

protein using ^{125}I -hydroxybenzylpindolol binding), not blocked by cyclohexamide, and decrease of AC in PL, SR and M. (ii) An aqueous sciatic nerve factor, as a putative trophic/regulatory factor, applied in vitro at higher concentrations decreased βAR of PL 30-40% in denervated muscle and 10-20% in normal muscle. (iii) Developmentally, in the rat βAR and AC are present from 18-day embryonic states (birth occurs at 21 days) but are not functionally coupled until 3 days after birth; this study was presented. (iv) Rat fast-twitch (FT, EDL) muscles and slow-twitch (ST, soleus) muscles showed differences in their PL AC and βAR activities. In intact muscles catecholamines induced greater production of cAMP in FT than in ST muscle. (b) Human muscle. (i) The subcellular distribution of βAR in normal human muscle was defined and presented. The distribution, $\text{PL} \gg \text{SR} > \text{M}$, correlated with our previous localization of AC in human muscle, supporting a functional coupling. (2) Guanylate cyclase (GC). (a) Animal muscle, (1) We previously reported increase of GC in all fractions (PL, SR, M and 10^5 g supernatant) of denervated rat muscle. We now find the increase is not blocked in cyclohexamide-treated animals, suggesting a mechanism other than new synthesis of GC must be responsible for the increase. (ii) Concanavalin A (Con A) inhibited particulate GC much more in rat denervated muscle than in normal muscle. (b) Human muscle. (i) In normal human muscle the subcellular distribution and properties of GC were similar to those we observed in rat muscle. The specific enzyme activity among subcellular fractions was $\text{PL} > \text{SR} > \text{M} > \text{soluble fraction}$ (10^5 g supernatant). Even though PL possessed the highest specific activity, the predominant portion of GC (60%) was in the soluble fraction. The enzyme required Mn^{2+} , but Mg^{2+} could substitute to a considerable extent. Also demonstrated were elution profiles on sepharose columns, substrate dependency, ionic requirements and pH profiles. (ii) Sedimentation profiles of human and rat PL and cytosol GC on 5-20% linear sucrose gradients were determined. PL GC sediments at $S_{20,w}$ of 11.2 while the cytosol gc at 9.1.

(3) Con A Binding greatly increased in denervated rat muscle PL and SR compared to controls. Kinetics of Con A binding PL and SR were determined. (4) Vanadate showed dual effects on PL AC. It activated basal AC but inhibited the activation by NaF and catecholamines.

Significance to Bio-Medical Research and the Program of the Institute:

These findings provide new information on the pathologic and pathogenic aspects of the various myopathies, on the treatment of some, and on animal models of some.

Proposed Course of Project: The studies underway are part of a long-term project consisting of interrelated investigations which will continue for several years.

Publications:

Askanas, V., Valle, D., Kaiser-Kupfer, M.I., Engel, W.K., Blumenkopf, B.: Cultured muscle fibers of gyrate atrophy patients: tubules, orithine toxicity, and l-ornithine-2-oxyacid aminotransferase deficiency. Neurology 30: 368, 1980.

Askanas, V., Engel, W.K., Reddy, N.B., Barth, G., Bethlem, J., Kraus, D. R., Hibbard, M.E., Lawrence, J.V., and Carter, L.S.: X-linked recessive congenital muscle fiber hypotrophy with central nuclei. Abnormalities of growth and adenylate cyclase in muscle tissue cultures. Arch. Neurol. 36: 604-609, 1979.

Engel, W.K. Dagen des Oordeels: pathokinetic mechanisms and molecular messengers (a dramatic view). Arch. Neurol. 36: 329-339, 1979.

Zweig, M.H., Adornato, B.T., Van Steirteghem, A.C., Engel, W.K.: Serum creatine kinase BB and MM concentrations determined by radioimmunoassay in neuromuscular disorders. Ann. Neurol. 7: 324-328, 1980.

Lavenstein, B., Engel, W.K., Reddy, N.B., Carroll, S.: Autoradiographic visualization of β -adrenergic receptors in normal and denervated skeletal muscle. J. Histochem. Cytochem. 27: 1308-1311, 1979.

Engel, W.K., Prockop, L.D., Askanas, V., Engel, A.G., Hutchinson, R., Galdi, A.P., Williams, J., Goldman, A., Foster, L.: Nearly-fatal lipid-laden myopathy with myoglobinuria and myodeficiency of carnitine: prednisone-failure but dramatic improvement with carnitine, and cultured muscle dependence on carnitine. Neurology 30: 368, 1980.

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Reddy, N.B. and Engel, W.K.: In vitro characterization of skeletal muscle β -adrenergic receptors coupled to adenylate cyclase. Biochim. Biophys. Acta. 585: 351-354, 1979.

Reddy, N.B., Oliver, K.L., Engel, W.K.: Developmental patterns of adenylate cyclase and β -adrenergic receptor in rat skeletal muscle. Fed. Proc. 38: 843, 1979.

Foidart, M., Foidart, J.M., Engel, W.K.: Collagen localization in normal and fibrotic human striated muscle: an immunofluorescence study. Arch. Neurol., 1980, in press.

Askanas, V., Worthington, E.K., Engel, W.K. and Cunningham, G.G.: Ultrastructural localization of non-specific esterase in normal and pathologic human muscle. Trans. Soc. Neuroscience, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01190-16 MN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Myasthenia Gravis (MG)		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: W. King Engel, M.D., Chief, Neuromuscular Diseases Section, NINCDS OTHER: Valerie Askanas, M.D., Ph.D., Associate Neurologist, MNB, NINCDS Marinos C. Dalakas, M.D., Assistant Neurologist, MNB, NINCDS John Rose, M.D., Clinical Associate, MNB, NINCDS Charles McIntosh, M.D., Surgery Branch, NHLBI Allen S. Lichter, M.D., RO, COP, DCT, NCI Israel Yaar, M.D., Clinical Associate, MNB, NINCDS Alan Goldstein, Ph.D., George Washington U. Bruce T. Adornato, M.D., Palo Alto Med. Clinic, CA John McClure, Ph.D., George Washington U. Marguerite Foidart, M.D., MNB, NINCDS		
COOPERATING UNITS (if any) Surgery Branch, NHLBI; RO, COP, DCT, NCI; George Washington University, Washington, D.C.; Palo Alto Medical Clinic, CA		
LAB/BRANCH Medical Neurology Branch		
SECTION Neuromuscular Diseases Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) To apply clinical, immunologic, tissue-culture, histochemical, pharmacologic, electrophysiologic, autoradiographic, radionuclide-scanning, and electronmicroscopic techniques to investigate the etiology and pathogenesis of <u>myasthenia gravis</u> . Especially, to seek new or improved methods of treatment.		

Project Description:

Objectives: To apply clinical, immunologic, tissue-culture, histochemical, electronmicroscopic, pharmacologic, electrophysiologic, autoradiographic and radionuclide-scanning techniques to investigate the etiology and pathogenesis of myasthenia gravis. Especially, to seek new or improved methods of treatment and diagnosis.

Methods Employed: A variety of basic and clinical investigative techniques, v.i., were applied to patients with myasthenia gravis and other disorders of neuromuscular transmission, and to induced animal-models thereof.

Patient Material: Myasthenia gravis patients, and patients with other disorders of neuromuscular transmission, participated in the investigative studies and therapeutic trials. Sera, muscle and thymus were obtained during diagnostic or therapeutic procedures.

Major Findings:

Myasthenia gravis (MG) is an acquired disorder affecting transmission at the neuromuscular junction, mainly in adults and older children. The primary cause is not known, but the pathogenic mechanism is considered to be dysimmune (or autoimmune). Untreated patients usually are seriously handicapped and many die. Palliative treatment with anticholinesterases and anti-pathogenic treatment, consisting of thymectomy, ACTH and more recently, prednisone, have helped considerably, but much disability, some fatality, and drug side-effects do occur.

A. Therapy.

1. Thymectomy. Last year we discussed our review of 55 consecutive thymectomies done over the past 10 years, which showed that thymectomy is potentially beneficial in all patients with onset in teen-ager or later, and repeat thymectomy can be remarkably beneficial in patients previously improved who subsequently exacerbate and do not respond to medical management. However, not all patients respond satisfactorily, and so other treatments are needed.

2. Prednisone. Long-term high-single-dose alternate-day prednisone (LT-HSDAD-Pred) was introduced to this disease by us 10 years ago, and one child was begun on treatment 14 years ago. Many patients continue to have excellent benefit. However, virtually all responders are, even with very gradual tapering of the LT-HSDAD-Pred, dependent on it, requiring 5-20 mg q.o.d. Prednisone in higher doses reduces circulating lymphocytes ($T > B$), and lymphocyte-response to mitogens ($T\text{-mitogens} > B\text{-mitogens}$), but those effects last no longer than 24-48 hours. Because prednisone has toxicity and because some patients do not respond satisfactorily to it, we are seeking other drugs to be used with prednisone or alone. We have found that azathioprine 3 mg/kg/d added to prednisone seems to provide better control in some MG patients.

3. New prednisone-responsive disorders. To be reported are three patients we have identified who, by detailed investigation, do not have one of the neuromuscular diseases (MG, dysschwannian polyneuropathy, dermatomyositis/polymyositis) known to respond to LT-HSDAD-Pred. Because their severe weakness was out of proportion to minimal or no histochemical and electrophysiologic abnormalities, LT-HSDAD-Pred was tried. Remarkable improvement was achieved in each, with return of weakness each time the dosage was lowered too much. They thus have a new disorder(s).

4. Splenic Radiation. Because lymphocytes are the circulating cells most sensitive to x-irradiation and the spleen harbors a large amount of lymphoid tissue, low-dose splenic radiation was introduced as a treatment of MG patients resistant to all other forms of therapy (with RO, NCI) Three of five patients benefited objectively, with no serious side-effects. The benefit was transient (weeks to months).

5. Poly-ICLC. We have found this to be a new anti-dysimmune drug (see ALS and Peripheral Neuropathy project). One MG patient showed apparent improvement on a very short course and one on a longer course showed no response; more patients will be studied.

B. Pathogenesis. Questions regarding pathogenesis include possible altered host (patient) immunologic response, "foreigned" host cells, exogenous agent, role of thymus, role of thymus-produced peptide factors, role of lymphocytes, and role of nicotinic acetylcholine receptor (nAChR).

1. Thymus pathology. The rationale for the empirically-observed benefit of thymectomy (v.s.) is still being sought. In addition to the hyperplastic and thymomatous thymuses, we have now demonstrated that thymuses of older patients considered "atrophic" by pre-existing histopathologic criteria have evident in our fresh-frozen sections many small nests of lymphocytic and epithelial cells that look active -- we have postulated they may have a pathogenic role on the older non-thymomatous MG patient, and so have studied their chemical properties.

2. Thymic epithelial cells. a. Nicotinic acetylcholine receptor (nAChR) -- We have previously demonstrated nAChR in thymic epithelial cells histochemically with peroxidase-labelled α -bungarotoxin, and postulated that the thymic epithelial-cell nAChR may be the molecule foreigned by a putative exogenous agent. b. Thymosin -- This is a thymic hormone capable of repairing, sustaining and facilitating functions of lymphocytes. We have now reported the first cytolocalization of thymosin utilizing antibodies to synthetic thymosin α_1 -- it is in thymic epithelial cells of normal thymuses and of hyperplastic, "involted", and thymomatous (i.e. "benign" neoplastic proliferation of epithelial cells) thymuses of MG patients (with George Washington U.). A possible facilitatory role by thymosin produced by the active epithelial cells of MG thymuses in the pathogenesis of MG was postulated. c. Thymic epithelial cell cultures - cultured thymic epithelial cells from normal and MG human thymuses never developed myofibrils; they all had typical desmosomes and tonofibrils, and they formed typical Hassall's

corpuscles. Now we have presented that they (and not fibroblasts) contain thymosin by fluorescent antibody reaction (with George Washington U.). We are currently measuring the amount of thymosin they secrete in culture and will then study regulating mechanisms. We are now seeking evidence of nAChR in the cultured thymic epithelial cells with α -bungarotoxin by electronmicroscopic-cytochemical fluorescence and autoradiographic means.

3. Lymphocytes. a. Thymosin -- with antibody against thymosin α_1 , we have shown thymosin on/in circulating lymphocytes. In MG there is a higher percent of T-lymphocytes positive than normal, and this number is reduced after thymectomy of MG patients. The significance is not known. b. nAChR -- attempts to demonstrate this on thymic T-lymphocytes and circulating lymphocytes of normals, MG patients, and other neuromuscular diseases were negative using autoradiography, scintillation counting, and fluorescence microscopy with α -bungarotoxin labelled in various ways. c. Suppressor cells -- one hypothesis is that MG is caused by a defect of suppressor T-lymphocytes. We are studying the functional suppressor cells in MG, before and after thymectomy.

4. Hemopexin. We reported last year that serum hemopexin is increased in MG patients, and have now shown that it is due to the much-increased hemopexin synthesis exceeding the slightly-increased hemopexin catabolism. The hemopexin elevation in MG was inexplicable until we found increased myoglobin in the serum of MG patients by use of a very sensitive complement-fixation technique. Together, the findings suggested a small amount of myoglobin leakage may result from a hitherto overlooked minimal subclinical plasmalemmal-leaking myopathy in many MG patients.

Significance to Bio-Medical Research and the Program of the Institute: These findings present new information on the pathologic and pathogenic aspects of myasthenia gravis, and other defects of neuromuscular transmission, on treatment, and on corresponding animal-models.

Proposed Course of Project: To develop more fully the interlinked basic and clinical studies underway directed toward clarification of the pathogenesis and identification of the etiology, and toward elaboration of better means of treatment and prevention.

Publications:

Engel, W.K., Dalakas, M.C., Lichter, A.S.: Intractable myasthenia gravis can respond to splenic radiation. Neurology 30: 389, 1980.

Dalakas, M.C., Engel, W.K., McClure, J.E., Goldstein, A. L., Askanas, V.: Localization of thymosin α_1 in thymic epithelial cells of normal and myasthenia gravis patients and in thymic cultures. Neurology 30: 388, 1980.

Dalakas, M.C., Engel, W.K., McClure, J.E., Goldstein, A.L.: Thymosin α_1 in myasthenia gravis. New Engl. J. Med. 302: 1092-1093, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01189-12 MN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Episodic Weakness and Myotonic Disorder		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: W. King Engel, M.D., Chief, Neuromuscular Diseases Section, NINCDS OTHER: N. Bojji Reddy, Ph.D., Guest Worker, MN, NINCDS Valerie Askanas, M.D., Ph.D., Associate Neurologist, Medical Neurology Branch, NINCDS Albert J. Tahmoush, M.D., Clinical Associate, MN, NINCDS Marinos Dalakas, M.D., Asst. Neurologist, MN, NINCDS G.K. Bergely, M.D., NICHD P. G. Nelson, M.D., NICHD M. Jackson, Laboratory of Biophysics, MN, NINCDS B. Joshi, M.D., Clinical Associate, MN, NINCDS Lillian Recant, M.D., VA Hospital, Washington, D.C.		
COOPERATING UNITS (if any) LB, NINCDS Laboratory of Developmental Neurobiology, NICHD VA Hospital, Washington, D. C.		
LAB/BRANCH Medical Neurology Branch		
SECTION Neuromuscular Diseases Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.5	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less, - underline keywords) To define more clearly and to treat those disorders affecting the neuro- muscular apparatus which present primarily with <u>episodic weakness</u> or paralysis or are characterized by a significant amount of <u>myotonia</u> , i.e., conditions in which the main site of intermittent dysfunction is somewhere within the muscle fiber plasmalemma, T-system, sarcoplasmic reticulum, or myofibrillar complex -- i.e., the total excitation-contraction coupling mechanism. With respect to <u>periodic</u> <u>paralysis syndromes</u> , studies are done with agents which are transiently either therapeutic or provocative, with a view to obtaining more information regarding abnormalities of pertinent metabolic pathways and methods of treatment. The various <u>myotonia disorders</u> are studied with respect to more clearly defining the molecular abnormalities, seeking the underlying pathogeneses and treatment there- of, and finding better ways of symptomatically treating their myotonia. Induced animal-models of myotonia are also used for these purposes. Tissue culture of the human abnormal muscle is used to reincarnate the disease in culture and thence its		

analysis, e.g., with electronmicroscopy, biochemistry and microelectrodes, and its treatment; cultured human or animal muscle is also used for induction of models of diseases in vitro by chemical agents.

Project Description:

Objectives: To define more clearly and to treat those disorders affecting the neuromuscular apparatus which present primarily with episodic weakness or paralysis, or are characterized by a significant amount of myotonia, conditions in which the main site of intermittent dysfunction is somewhere within the muscle fiber plasmalemma, T-system, sarcoplasmic reticulum, or myofibrillar complex -- i.e., the total excitation-contraction coupling mechanism. With respect to periodic paralysis syndromes, studies are done with agents which are transiently either therapeutic or provocative, with a view to obtaining more information regarding abnormalities of pertinent metabolic pathways and methods of treatment. The various myotonia disorders are studied with respect to more clearly defining the molecular abnormalities, seeking the underlying pathogeneses and treatment thereof, and finding better ways of symptomatically treating their myotonia. Induced animal-models of myotonia are also used for these purposes. Tissue culture of the human abnormal muscle is used to reincarnate the disease in culture and thence its analysis, e.g., with electronmicroscopy, biochemistry and microelectrodes, and its treatment in vitro; cultured human or animal muscle is also used for induction of models of disease in vitro by chemical agents.

Methods Employed: Various techniques of clinical investigation including electromyography, clinical biochemistry, and muscle biopsy with samples for histochemical analysis, electronmicroscopy, tissue culture and biochemical assays were utilized. Cultured muscle was studied with various techniques, including intracellular microelectrodes, electronmicroscopy and biochemistry. Therapeutic trials to raise or lower potassium or sodium and provocative loading tests were used. Acetazolamide or dichlorphenamide was administered as a prophylactic agent for hypokalemic periodic paralysis, and as a treatment of myotonia. Diazacholesterol, a myotonogenic agent, was administered to human and animal muscle growing in culture.

Patient Material: Patients of all ages are admitted to the Medical Neurology Branch for this project if they have: intermittent muscular weakness associated with familial periodic paralysis, hypo- or hyperkalemic; isolated examples of periodic paralysis with potassium disturbances; thyrotoxic periodic paralysis; paramyotonia congenita; myotonia congenita; or myotonic atrophy. (Patients with myasthenia gravis are part of another project.)

Major Findings:

1. Periodic Paralysis (PP). These are hereditary or acquired disorders causing chronic weakness punctuated by attacks of paralysis. There are potassium-benefited and potassium-provoked forms. Associated metabolic abnormalities are known but the actual pathogenic mechanisms are not. Standard palliative/preventive therapy in the idiopathic hypokalemic form of PP

is potassium, more recently acetazolamide, and most recently dichlorphenamide. 1. Treatment. In the hypokalemic form of PP, the treatment we introduced, long-term acetazolamide, has continued to be the best prophylactic agent both for preventing attacks and improving inter-attack weakness. It is now in the textbooks as such. Two of our patients have been treated successfully for more than 14 years. In hypokalemic PP patients not responding to, or worsened by, acetazolamide, we have recently found that dichlorphenamide, another carbonic anhydrase inhibitor, can be dramatically beneficial in improving erstwhile "permanent" weakness; these patients may be a separate subset. Since human muscle contains either no, or very little (per disparate studies of ourselves cf. others) carbonic anhydrase, the mechanism of acetazolamide and dichlorphenamide benefit in hypokalemic PP remains unknown. 2. Pathogenesis. Muscle of patients with hypokalemic PP was grown aneurally in tissue culture to obtain fibers free of all neural, circulating and other influences existing in the patient. No abnormalities of growth pattern or light- or electron-microscopic appearance was detectable. Cultured fibers of PP patients will be studied with microelectrodes for plasmalemmal biophysical properties (v.i.).

IIA. Myotonia Congenita and Paramyotonia Congenita. Myotonia is a crippling symptom in these inherited diseases of unknown causes. (1) Clinical Studies. Two years ago we reported acetazolamide as a new treatment providing excellent benefit in patients who had failed to respond to other anti-myotonia agents. It continues to have long-term effectiveness in those and several additional patients. 2. Pathogenesis. Growth in culture of muscle from myotonia congenita patients is normal by light and electronmicroscopy. Biophysical properties of plasmalemma of those cultured fibers is mentioned below.

IIB. Myotonic Atrophy (Myotonic "Dystrophy"). This is an inherited multisystemic disease, with progressive muscle weakness and wasting, of unknown pathogenesis. We have previously raised the possibility of at least a partially neurogenic aspect. With our new concept of "myogenous dys-innervation" we have now extended that hypothesis to include a possible myogenous muscle plasmalemmal non-receptivity to neural short- and long-term trophic influences. (1) Pathogenesis, patient studies. (a) Growth in culture -- muscle fibers cultured from the patients show normal growth and appearance by light- and electron-microscopy. (b) Plasmalemmal biophysical properties of cultured human muscle -- with microelectrodes (with NICHD) we have successfully obtained baseline values in 37 normal fibers: resting membrane potential 51.5 ± 4.6 mV, input resistance 11.6 ± 5.9 M Ω L; only 2 of 34 fibers electrically excitable at RMP, but when hyperpolarized to -80 mV an action potential could be elicited from all with threshold for excitation at 19.3 ± 4.9 mV and action-potential amplitude of $91.7 \pm$ mV, with repetitive trains of action potentials occurring after anodal break. Against these we have now compared 42 fibers cultured from patients with myotonic atrophy and found no difference. A future step will be to study the effect on these parameters of motor neuron innervation in vitro of the cultured human fibers, because, based on these results, we have postulated that in myotonic atrophy an intrinsic muscle-cell abnormality may require motor innervation and/or a

circulating factor for full expression. In the cultured myotonic muscle we will also study chloride and other ionic conductances and ionic dysequilibrium challenges. We have also measured in cultured normal human muscle ion channels responsive to applied acetylcholine and values different from rat cultured muscle were found; these will next be studied in cultured human myotonic and periodic paralysis muscle (with LB). (c) Insulin receptors on leucocytes are being studied. (2) Pathogenesis, animal models. Diazacholesterol-induced myotonia in the intact animal we discussed last year, including the lowered adenylate cyclase of muscle. We have now presented a report on the effects of diazacholesterol on cultured rat muscle. The drug caused: change of spontaneous contraction rhythm to more continuous and fibrillation-like movements; electronmicroscopically evident smeared z-discs, disorganized myofibrils, some honey-comb appearance and dilation of sarcoplasmic reticulum, and reduced concanavalin A binding to the plasmalemma; increased mobility of ^{125}I -Con A biochemically; 40-50% decreased adenylate cyclase (basal, and NaF and isoproterenol stimulated) but normal density and affinity of β -adrenergic receptors, suggesting uncoupling of the latter from adenylate cyclase; and increased guanylate cyclase and cGMP-phosphodiesterase, indicating a greater turnover of cGMP.

Significance: These findings present new information on the pathologic and pathogenic aspects of the periodic paralyses and the disorders with myotonia, on their treatment, and on corresponding animal-models.

Proposed Course of Project: To explore in more detail, with patients and animals, the mechanism of action of acetazolamide and dichlorphenamide prophylaxis in hypokalemic periodic paralysis and the pathogenesis of the disease itself. To seek even better therapeutic agents. To explore the underlying nature of myotonia and the method of its benefit from acetazolamide and to seek improved methods of treating myotonia and the underlying disorders.

Publications:

Tahmoush, A.J., Bergey, G.K., Askanas, V., Nelson, P.G. and Engel, W.K.: Electrical properties of aneurally cultured adult human muscle. Trans. Soc. Neuroscience. 760, 1979.

Tahmoush, A.J., Askanas, V., Nelson, P.G. and Engel, W.K.: Electrophysiologic properties of aneurally cultured muscle from myotonic atrophy patients compared with controls. Neurology 30: 103, 1980.

Dalakas, M.C. and Engel, W.K.: Treatment of "permanent" muscle weakness in hypokalemic periodic paralysis. Neurology 30: 103, 1980.

Jackson, M., Lecar, H., Askanas, V. and Engel, W.K.: Single acetylcholine channels in cultured human muscle. Trans. Soc. Neuroscience, in press, 1980.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Surgical Neurology Branch

National Institute of Neurological and Communicative Disorders & Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980
Surgical Neurology Branch, IRP
National Institute of Neurological and Communicative
Disorders and Stroke

Paul L. Kornblith, M.D., Chief

Summary of Studies in the Surgical Neurology Branch

This annual report is the second of the newly reorganized Surgical Neurology Branch beginning October 1, 1979. This period of time has been occupied by ongoing reorganization and refurbishing of the research and clinical facilities of the Surgical Neurology Branch. Over the past year both the research and clinical programs of the Branch have continued to develop and become fully productive. The electron microscopic and image analysis facilities of the Branch have now been largely completed and the programs dependent on these set into motion. The tissue culture facilities have seen high utilization and their expansion is now underway. Twenty new human brain tumor lines are now under detailed study. Others brought from Massachusetts General Hospital continue to be utilized for both immunological, chemotherapy and basic cell biological studies of human gliomas and other CNS tumors. New research programs in cellular immunology, humoral immunology and antigenic purification for gliomas have been set up and are now fully active and productive.

Over the year some one hundred surgical cases have been done, with many of these providing new tumor material for study in the laboratories. A major upgrading of the surgical facilities is continuing with the installation of the positron emission tomographic scanner. Metabolic studies of patients with brain tumors have been initiated as well.

The primary areas of our research activities have included:

1. Biological, immunological and chemotherapeutic studies in human brain tumors.
2. Biological and immunological factors in peripheral nerve injury, neoplasia and regeneration.
3. Biological studies of human pituitary tumors.
4. Neurodiagnostic studies including the PECT scan.
5. Neurophysiological studies.

Clinical protocols are now approved and underway. These are:

1. Evaluation of Biological, Immunological and Chemotherapeutic Parameters in Brain Tumor Patients (79-N-89)

2. Immunotherapy of Malignant Brain Tumors (79-N-133)
3. Biological Studies of Human Pituitary Tumors (79-N-151)
4. Evaluation of Biological and Immunological Parameters in Peripheral Nerve Regeneration (80-N-06)
5. ^{18}F -2-Fluoro-2-deoxy-D-Glucose (FDG) Positron Emission Computed Tomography (PECT) in Typing of Cerebral Gliomas (80-N-36)
6. ^{18}F -2-Fluoro-2-deoxy-D-Glucose (FDG) Positron Emission Computed Tomography (PECT) in Epilepsy (8-N-58)
1. BIOLOGICAL, IMMUNOLOGICAL AND CHEMOTHERAPEUTIC STUDIES IN HUMAN BRAIN TUMORS

A. Biological Studies

In order to determine the ways in which human brain tumors will behave in a given patient, it is necessary to have cellular models of their biological activity. Such cellular models are provided by the tissue culture system. In this system it is possible to grow approximately 90% of human brain tumors in an environment outside of the human body. Utilizing this approach we have been able to show that certain characteristics of cultured human brain tumor cells not only parallel those of the cells in a patient but also offer us the opportunity to add therapeutically relevant information to the planning of optimal therapy and the prediction of the way in which a tumor will grow in a given patient. This type of work has two major areas. First is the area of the prediction of the behavior of tumors which are known to be malignant. Here the major question is how malignant a given tumor will be. Secondly, in certain tumors which by and large are benign or nonmalignant in their growth, there are occasional instances in which tumors do grow in a malignant fashion. In the second category, therefore, the question is being able to pick out ahead of time those tumors which behave in a malignant or invasive fashion. These are the two primary goals of the program in the study of tumor biology. There are, in addition, several secondary goals. These include: studies of the basic biologic mechanisms of tumor growth and the similarities and differences of this tumor growth to the growth of normal cells. In order to achieve successful evaluation of all of these primary and secondary goals we have established a tissue culture laboratory at the NIH for the study of human brain tumors.

Of major importance for these studies has been the building of our electron microscope laboratory (under Dr. Barry Smith's direction) which now includes two transmission electron microscopes, a scanning microscope, x-ray spectroscopy, and

image analysis capabilities. This facility is in operation now. Further testing and adjustment of some of the more technologically sophisticated x-ray and image analysis equipment continues but the primary electron microscope functions are fully active. General EM transmission characterization of 20 glioma lines is being carried out in addition to studies of special surface staining properties. Scanning microscopy of isolated tumor cells and tumor cells interacting with lymphocytes will allow visualization of tumor to immune cell interaction.

In addition to equipment development, the SNB scientific team devoted to this area has also grown. Since the last Annual Report, Dr. Maurice Gately and Dr. Nobuyuki Shitara have joined the Surgical Neurology Branch staff. Dr. Gately, a cellular immunologist, has set up the SNB cellular immunology laboratories and begun a major study of specific and non-specific lymphocyte-mediated tumor cell cytotoxicity. Dr. Gately also has added Dr. Moshe Glaser, a cellular immunologist, to this staff as an expert. Dr. Shitara, a Visiting Scientist to be with us for two years, has brought techniques of flow cytometric DNA analysis that have been applied to the characterization of approximately 20 glioma cell populations. He has also developed an assay for glycoproteins released by glioma cells. He has found one glycoprotein of 85,000 MW that is specifically released by C6 glioma cells. This is now being tested for immunological properties as well as possible tumor growth regulatory functions. Dr. Paul McKeever, the neuropathologist who joined the SNB in July of 1979, has similarly built a new laboratory facility and team which has been very productive over the period of this Annual Report. Working on characterization of glioma cells, this team has studied cytoplasmic and "released" proteins of human glioma cells as well as the inhibition of release of such proteins by 3'-5' cyclic adenosine monophosphate (cAMP). The latter is the first example for any cell system that cAMP can be inhibitory to cellular release of proteins. In all other systems it is excitatory. In addition, if such release can be blocked, it may have important implications for control of glioma tumor cell growth.

Dr. McKeever has also provided neuropathological classification of SNB tumors both in operating room specimens and in subsequent tissue culture. He together with Dr. Barry Smith and electron microscopy support staff has begun an in depth ultrastructural characterization of available glioma lines including inter-cellular junctions, glial filaments, surface membrane parameters, nuclear morphology, and so forth. Since precise characterization of the cells from tumors and in culture is so critical to all the work for the SNB, the establishment of this capability is a major step forward for the SNB. A further important advance for characterization has been the institution of a glial fibrillary acid protein (GFAP) assay system under Dr. Eugene Quindlen's direction. Anti-human GFAP antibody is now being prepared and will be used to determine the presence or absence of GFAP in the glial tumors and cell lines.

Each of these specific advances in biological characterization fit into our overall ongoing program of glioma cell analysis. These ongoing studies include analyses of the rate of growth of individual as well as populations of tumor cells; the ability of tumor cells to grow under stressful condition such in soft agar or in low serum concentrations; their ability to invade neighboring tissue; their chromosomal pattern and abnormalities; and their growth and malignancy in animal hosts. This latter technique is perhaps the most crucial in determining whether an individual human tumor can reproduce via tissue culture a tumor in the in vivo situation.

These studies of tumor malignancy are supplemented by studies of tumor characterization in which tumor cell populations are characterized as to their cell or cells of origin. It is extremely important in deciding whether or not a given tumor will behave in a malignant or in a benign fashion, relatively speaking, to determine what type of cell predominates. Frequently, human brain tumors are of mixed cell origin or at least mixed cell origin as regards the degree of malignancy of the cell comprising the tumor mass. Our tumor characterization includes detailed studies of cellular ultrastructure membrane and cytoplasmic biophysical properties and biochemical properties, such as S-100 protein, the enzyme cortisol acetyltransferase and glial fibrillary acidic protein (See above). In addition, studies of myeline basic protein and cerebral gangliosides may be of importance. Such studies have been initiated on the tumor cell lines under evaluation.

From all these biological studies it appears possible (as we have reported previously) to determine the degree of malignancy and to characterize the cell of origin of a given tumor. This data can be useful in determining the prognosis of the individual patient and also in the planning of individualized, optimal therapy. For example, it is possible to assess the likely responsiveness to radiation therapy from the type of kinetic growth pattern of a particular tumor. The most rapidly growing tumors in tissue culture are the most sensitive to radiation. Likelihood and rapidity of recurrence may also be determined.

B. Immunological Studies

It is highly likely that immunotherapy will be an important part of brain tumor therapy in the near future. With this in mind development of a systematic approach to the study of the immunological aspects of brain tumors both with respect to the tumor and the host has been undertaken. This includes the cellular as well as the humoral facets of immunological interaction. We have been extremely fortunate to have Dr. Eugene Quindlen, a skilled humoral immunologist, as well as Dr. Maurice Gately, an excellent cellular immunologist, join our group. We thus have special expertise in the two crucial areas of immunological interaction in human tumors. We have established a laboratory for the study of immunology which includes extensive

technology for the separation of immunoglobulins, for the characterization of immune globulin and antigen activity and for the characterization of cellular subpopulations of lymphocytes as well as the study of their interaction with brain tumor target cells. Further, we now have in force an immunotherapy protocol for glioma patients which is among the first three in the world.

The approach which we are using is to study the immune response in tissue culture utilizing individual patient's tumor cells as targets. The cultured tumor lines enable us to study the interaction in a very direct way with humoral factors and cellular factors studied either separately or in combination.

We have studied the cytotoxic antibody responses in the serum of all human patients with gliomas who have been operated on here at the NIH in this interval. Additionally, immune adherence techniques have been used to characterize the antibody response. The fractions responsible for the immune adherence response have been found to be largely IgM while both IgG and IgM are involved in the cytotoxic responses. These studies have indicated the feasibility of the determination of the system of antitumor immune responses in which the role of specific antibody fractions can be delineated. It may well be that the immune response is not purely one of blocking lymphocyte interaction with target cells but that the humoral response may in certain instances be helpful in destroying tumor cells. Such a conclusion is supported by the observation that the presence of cytotoxic antibody in the serum of patients does indeed correlate with longer survival. The fact that there is a difference between the cytotoxic and immune adherence antibodies is supported by the fact that there is marked difference between patients who have immune adherence responses and those who have cytotoxic responses and that the responses do not necessarily correlate directly.

The humoral immunological techniques have a potential direct diagnostic value. The detection of antibody activity in the serum of patients harboring brain tumors can be made in over 80 percent of such patients. A simple diagnostic test of immune response in individual patients for telling whether they may or may not have a brain tumor is thus a practical reality. More needs to be determined about the specificity of the response. To date we know that it is present in "normals" at the level of only 9 percent. Patients with metastatic tumors show a 30-40 percent positive response.

Tissue culture target glioma cells are crucial for our new immunotherapy program which can only be effective if there is an understanding of baseline immunological responses. We have looked for autologous cytotoxic antibodies by means of a complement-dependent microcytotoxicity assay in the serum of over thirty patients. The lower grades of astrocytoma, Grades I-III have a consistently higher rate of positive responses than do the Grade IV's. It also seems that the presence of cytotoxic antibody correlates with longer survival. Autologous fibroblasts

were also tested in fifteen patients. All fibroblasts were negative for cytotoxic antibody supporting the fact that there is some degree of tumor specificity for this serological assay.

We have begun the initial phases of our immunotherapy program and are presently dealing with the question of growing and maintaining cells in autologous human serum without addition of Fetal Bovine serum. We have successfully injected a primate with irradiated human tumor cells. We are almost ready to inject our first patient to try and enhance immune response in dealing with their own tumor.

We have also begun to investigate the different variables which may modulate immunological competence. Some of these include calcium, the steric relationship of the tumor itself and immuno-modifiers such as cAMP, Con A, anesthetics and Atromid-S. If we are able to understand the mechanisms of these variables, it may be possible to modify immune response in vivo by modifying their interaction.

We have also been looking for antiglioma antibodies in sera from families where one or more members have neurofibromatosis. Preliminary results are somewhat encouraging and show a possible positive correlation between presence of antibodies and clinical disease. Further investigation will be necessary to determine how precise this correlation is and whether there is predictive value in our assay for siblings who do not yet show clinical signs of neurofibromatosis.

The major goal of our next phase of work will be to begin patients in the immunotherapy program. We will be pursuing our efforts simultaneously to further define and modulate the humoral immune response.

C. Chemotherapy Studies

At the present time the major therapeutic modality being explored to alter the prognosis in human brain tumors is that of chemotherapy. The most effective forms of chemotherapy - the nitrosourea compounds - are effective in only 50 percent of patients. Therefore, it is important to determine which patients will respond to which agent. We have developed a system, again using our tissue culture approaches whereby the individual patient response can be determined. Using this response we have been able to show that there is a direct correlation between individual patient response to a given agent in their tissue cultures and their response clinically. This has necessitated the use of CAT scan follow-up well as clinical evaluation on a serial basis. Thus far, we have evaluated over 50 patients, only 14 of which met both in vitro and in vivo criteria for study. Six of nine cell lines (67%) responding to BCNU in vitro were shown to correlate with tumor sensitivity clinically, i.e., tumor size decreased in these patients. Five of five patients were shown neither to be sensitive in vitro nor in vivo (100%).

Currently, an additional series of patients is being evaluated for conformance to study criteria.

The observation that there is a correlation enables us to do more extensive studies in determining why there are or are not specific responders to chemotherapeutic agents. These studies of specific responders and nonresponders have included differences in membrane characteristics such as surface coating and membrane fluidity; in state of differentiation; in oxidative metabolism and in protein synthesis. Agents used for these studies include Concanavalin A, dibucaine, s-adenosyl-L-methionine, and peromycin. Collaborative studies of DNA strand break repair have thus far revealed that drug action is not limited to one mechanism alone. Preliminary study of the nitrosoureas indicates that cellular sulphydral concentration may be important. Ongoing studies are examining this area in greater detail. From these more extensive and sophisticated studies we may be able to learn what makes a cell or a given tumor respond or not respond to chemotherapy.

We are presently developing our model system for a more detailed and thorough study of how the drug interaction with the more commonly used agents (e.g., nitrosoureas, vincristine, procarbazine, methotrexate) as well as some of the newer agents can be evaluated on a patient by patient basis with the plan of developing a rational prospective chemotherapeutic plan and with the hope that agents or factors enhancing the response may be developed. Both cytotoxic and cell differentiation agents (e.g., cyclic adenosine monophosphate, dimethylformamide, DMSO, and hexamethylene bis acetamide) are being explored for their potential interactions leading to enhanced killing of neoplastic cells.

We are currently using a modification of the microcytotoxicity assay to test new chemotherapeutic agents for the National Cancer Institute. Many of these agents are not soluble in aqueous media, presenting the problem of solvent toxicity. The new solid-phase drug delivery system permits the evaluation of agents (e.g., PCNU, AZQ, Spirohydantoin and Rapamycin) that would otherwise be impractical in the microtiter system. Preliminary study has shown the effectiveness of some of these drugs as chemotherapeutic agents.

As part of an exploration of the effects of the various components of standard glioma therapy, we have examined the effects of phenytoin (used as a prophylactic anticonvulsant after glioma surgery) and found an inhibition of the growth of at least 50 percent of glial brain tumors. We have previously published human brain tumor data on this inhibition and have now documented these findings in two rat glioma tumor models (C6 and RT9). Acute and chronic survival experiments using the RT9 in a subcutaneous tumor model have been conducted. Consideration of dilantin metabolism kinetics has resulted in studies of staggered delivery schedules and the use of subcutaneously-implanted osmotic pumps.

In vitro experimentation with cultured TR9 and C6 tumor cells is being conducted with the microcytotoxicity assay. These experiments confirm our previous studies of murine tumor sensitivity to dilantin. Further work to elucidate the basis of the tumor growth-inhibitory effect of phenytoin is proceeding.

2. BIOLOGICAL AND IMMUNOLOGICAL FACTORS IN PERIPHERAL NERVE REGENERATION

A vein grafting technique for rat sciatic nerve was developed and perfected during the preceding year to serve as a model system for the study of molecular and cellular factors in neuronal regeneration after injury. The vein graft serves to provide a chamber into which various molecular factors such as collagen or growth factors (epidermal growth factor, nerve growth factor, fibronectin, etc.) or cells such as cultured fibroblasts, Schwann cells, central glial or glioma cells can be introduced to study their effects on axonal regrowth through the graft. Vein grafts alone, cut nerves alone, sutured reapproximated nerves and nerve grafts have been utilized as controls with the studies covering the post-injury period from immediately post-injury to six months.

The rat model studies to date have shown enhanced regeneration in the vein grafted nerves compared to injured but untreated nerves, which is in accord with previous studies of nerve regeneration. Both long- and short-term experiments have now been completed and are being analyzed. Inhibitory effects of microcrystalline collagen and previously noted immunological reactivity have been documented. Specific lectins also appear to have a negative influence on early regeneration. Human glioma cells injected into the graft appear, under certain conditions, to promote regeneration. The influence of fetal rat cell lines (various origins) is also being studied. Light and transmission and scanning electron microscopic techniques are being applied. A new image analysis computer system now installed will facilitate the data analysis.

Finally a clinical protocol for study of failed human peripheral nerve regeneration has been instituted and the first patients entered.

3. NEURODIAGNOSTIC STUDIES

RESEARCH IN THE "NEURORADIOLOGY AND COMPUTED TOMOGRAPHY SECTION"

The largest effort of the Neuroradiology and Computed Tomography Section research activity has been concentrated on computed tomography (CT) both in its transmission and emission (Positron Emission Tomography) modalities.

Computed Tomography (CT) in its transmission and emission modalities, represents the main research area of the Neuro-radiology and Computed Tomography Section.

Ongoing clinical-animal/experimental research projects in transmission CT include studies of degenerative, demyelinating and atrophic processes of the brain, hydrocephalus, brain edema, postradiation cerebral necrosis, surgically correctable lesions in young patients affected by chronic epilepsy, diseases of the spine and the spinal cord, attempts at tissue characterization of normal and abnormal (e.g. tumoral) cerebral tissue, and an experimental glioma model in primates.

Physics projects: improved dual-energy CT scanning using both a split-detector and a dual kVp method; analysis of aliasing effects and developments of methods for their elimination; phantom studies for the evaluation of artifacts and calibration of CT machines. Feasibility tests to build a new type of CT device which will use protons are under way.

Positron Emission Computed Tomography (PECT) allows us to obtain pictorial data (e.g., axial transverse or coronal images of the brain) as well as dynamic functional data (such as regional cerebral glucose consumption rate-mg/min/100 gm- of brain substance, measurements of the storage, degradation and turnover of tagged metabolites, follow-through of the movement of the CSF in the deep CSF intracranial cavities). The unique property of PECT is that it provides physiologic information not available with any other imaging procedure.

During the last year significant progress has been made in our Section on the design of a high-resolution high-sensitivity scanner for head and animal studies--the Neuro-PET.

Two clinical protocols for the PECT scanner have been instituted. Approximately 10 patients with gliomas have now been studied with 18-F-deoxyglucose. An epilepsy protocol has similarly been instituted.

Radionuclide ventriculography and cisternography are diagnostic tools permitting the morphologic and dynamic study of the cerebrospinal fluid pathways more accurately than has even been possible with any other diagnostic test.

The adjunction of positron emission computed tomography to our diagnostic armamentaria should improve significantly the information content of our radionuclide ventriculograms and cisternograms.

Selective arteriography (radiographic) of the spinal cord is a diagnostic technique which has proven to be very informative in cases of arteriovenous malformation, tumor, obstructive vascular disease, trauma, and postradiation damage of the spinal cord.

Radioisotope angiography of the spinal cord offers distinct advantages as a screening method, and in certain types of intraspinal pathology may give information not available by any other diagnostic test.

Preliminary experience with computed tomography of the spine after injection of contrast medium indicates that this methodology is useful in the evaluation of certain vascular lesions of the spinal cord.

Experimental spinal cord angiography in the rhesus monkey is increasing our understanding of the blood supply of the spinal cord both in physiological and pathological conditions. Recently this technique has been used as a basis for attempts at experimental surgical revascularization of the cord in primates. Microsurgical revascularization of the spinal cord is now being carried out in rhesus monkeys as part of this program.

4. NEUROPHYSIOLOGICAL STUDIES

Under the direction of Dr. Choh-Luh Li investigations of the neurophysiological mechanisms of pain and epilepsy have been carried out. Recent emphasis has been on pain mechanisms using a vagus nerve model. Extracellular and intracellular responses of the ganglion nodosum to stimulation of the vagus nerve were recorded. Based on the conduction velocity of these extra and intracellular responses, the ganglionic cells appeared to respond mainly to the impulses initiated from the C-fibers; and, occasionally also from the A-delta fibers of the vagus. Investigation of the electrical properties of the ganglionic cells (e.g., input resistance, capacitance, time constance, change in membrane potentials and discharge activities) in response to electric stimulation of the vagus and superficial peroneal nerves and to application of chemical agents is in process. At present, no conclusive statements can be made.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02367-02 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Biological, Immunological and Chemotherapeutic Studies of Human Brain Tumors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Paul L. Kornblith Other: Barry H. Smith Eugene A. Quindlen Maurice K. Gately Paul E. McKeever Moshe Glaser Nobuyuki Shitara	Chief Medical Officer Senior Staff Fellow Senior Staff Fellow Medical Officer Expert Visiting Scientist	SNB NINCDS SNB NINCDS SNB NINCDS SNB NINCDS SNB NINCDS SNB NINCDS SNB NINCDS
COOPERATING UNITS (if any) Division of Radiation Therapy, NCI		
LAB/BRANCH Surgical Neurology Branch		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p>In this study human <u>brain tumors</u> are evaluated in a <u>tissue culture</u> environment as to their basic <u>biological</u> behavior, their response to <u>chemotherapeutic</u> agents and the detailed <u>immunological</u> interactions between the host and the tumor. A primary goal of this work is to improve the <u>therapy</u> of patients by understanding the basic <u>cellular biology</u> of malignant human brain tumors.</p> <p>Over the course of this year major accomplishments have been 1) expansion of the biological characterization program to include a detailed study of tumor <u>protein secretions</u> and DNA and establishment of a scanning and transmission <u>electron microscopy</u> facility; 2) development of a new <u>surface chemotherapy assay</u> enabling the testing of new drugs; 3) establishment of a <u>cellular</u> immunological assay for <u>gliomas</u> and glioma antigen purification methods and; 4) initiation of an immunotherapy clinical protocol.</p>		

PROJECT DESCRIPTION:

I. OBJECTIVES: There are three primary objectives of this research program. The first objective is to determine how increased understanding of the cellular biology of human brain tumors can lead to a better development of therapeutic and prognostic approaches. The second objective is to determine how a detailed study of immunological parameters in human brain tumors can be used to develop diagnostic and immunotherapeutic modalities for these patients. The third objective is to develop a program for rational planning of chemotherapeutic usage based on individual patient response.

II. MATERIALS AND METHODS:

A. The primary method used for this project is that of the tissue culture of human brain tumor cells. In this technique cells removed at operation are placed in a medium consisting of F10 with 10-12% fetal calf serum. The tissue removed at surgery is minced to 1 mm chunks and then explanted into plastic Falcon bottles with appropriate amounts of the standard medium. After cellular outgrowth begins the medium is changed regularly and then cells are subcultured when needed with .25% trypsin. The cells are passaged and harvested to provide the basic cellular material for all of our other techniques.

A new primate glioma model developed in the laboratory of Dr. John Sever (utilizing JC virus from human prognosis multifocal leukoencephalopathy is now being used to extend the human studies.)

B. Biological techniques: The detailed characterization of the tissue culture cells requires performance of PPLO or mycoplasma testing to determine that the cells are free of contamination. It also required light and electron microscopy, biophysical and biochemical studies. The electron microscopic studies involve scanning, transmission, surface replica and x-ray spectrometry studies to evaluate the surface and intracellular characteristics of the tumor cells. Direct correlations can thus be made of surface characteristics with malignancy. Biophysical studies include microelectrode recordings with single cell determination of cell brain resting potential, time constant and cellular resistivity. The biochemical studies including analysis of S-100, glial fibrillary acidic protein, myelin basic protein gangliosides collagen synthase, released proteins and glycoproteins, and flow cytofluorometric separation of tumor DNA can be performed on cultures at varying time periods from initial explanation. Characterization of the degree of functional malignancy can be accomplished by means of using the cell's ability to grow in low serum (.5%), ability to grow in soft agar and to penetrate nucleopore filters. The cells may also be studies in an animal model system such as the immunosuppressed hamster to determine whether they are able to produce a tumor similar to that seen in the patient.

C. Immunological studies: Two basic types of humoral immunology studies have been carried out. The first involves a microcytotoxicity assay. In this microcytotoxicity assay cells are transferred from Falcon plastic flasks (in a suspension of approximately 30-50 thousand cells per cc) to the individual wells of a Falcon microtiter plate using a Terasaki syringe at an approximate 100 density of cells per well.

These cells are allowed to establish themselves for 12-18 hours and then immunological testing with antibody and complement is carried out. The antibody can be either whole prepared from serum or serum which has been fractionated into its globulin components. The complement used is a combination of human pooled serum or human cord serum with rabbit serum as a primary complement source. It is important that the rabbit serum be obtained from rabbits approximately 4 to 6 weeks of age. The complement preparation is added approximately 1 hour after the addition of the serum and the plates are then incubated for 18 hours at 37°. Finally, the plates are stained with hematoxylin-Giemsa and the cells counted. By careful arrangement of the cells in the plate, it is possible to analyze the effects of 4 to 6 individual patient sera on a line simultaneously. This approach allows for excellent statistical quantitation and determination of what is known as the cytotoxic index (C.I.). this index is essentially the ratio of cells that have been eliminated by the immunological interaction to those in the untreated control wells. A cytotoxic index of .2 or above is generally statistically significant. Precise statistical determinations are made on each set of observations. The counting of cells in the plates has now been automated using an image analysis computer and scanner.

Immune adherence testing is carried out with the use of antibody from specific patients, target cells and red blood cells. The antibody-coated red blood cells attach to the surface of a target cell and when one sees adherence of numbers of red cells to a give target cell it is considered to be a positive response. This technique has the advantage of allowing careful serial titer dilution and also permits absorption with various cellular or tissue components.

In planning a rational program of immunotherapy against grain tumors, knowledge concerning the ability of brain tumors to elicit cell-mediated immune responses and concerning the susceptibility of brain tumors to be destroyed by various cell-mediated immune mechanisms is critical. However, very little is known regarding these questions. During the past year a laboratory for studying cell-mediated immune responses to brain tumors has been established. The initial goals in this work have been (1) to develop assays for monitoring the cellular immune status of brain tumor patients prior to and during immunotherapy and (2) to develop in vitro model systems for studying the ability of brain tumors to elicit and be destroyed by cell-mediated tumor destruction exist, including lysis of tumor

cells by specifically-immune cytolytic T lymphocytes (CTL), by natural killer cells (NK cells), by activated macrophages, and by antibody-dependent killer lymphocytes (K cells). Our initial work has focused on the potential role of specific cytolytic T lymphocytes in cell-mediated immunity to brain tumors. The possible importance of other cellular immune effector mechanisms will be examined in the future. Dr. Maurice Gately, who is directing this work, has previously had extensive experience in studying the generation and mechanisms of action of cytolytic T lymphocytes and in studying lymphokines, the soluble mediators of cellular immunity. Thus the present work represents a direct extension of his prior interests.

The initial requirement in these studies was to develop a reliable and quantitative assay for measuring cell-mediated immune lysis of human glioma cells. The ^{51}Cr well, and the rate of spontaneous release of ^{51}Cr from all glioma cell lines studied thus far has varied from 20 to 30% of the total releasable ^{51}Cr in 24 hours. Thus it is readily possible to use ^{51}Cr release to assess the ability of lymphocytes to lyse glioma cells over a 24 hour period.

Lymphocytes are isolated from human peripheral blood by centrifugation of Ficoll-Hypaque. This is followed by further centrifugation on sucrose gradients to remove contaminating platelets. Cytotoxic lymphocytes are generated by incubation of responder and stimulator lymphocytes and glioma cells in 2 ml volumes of culture medium containing 5% human AB serum in the wells of Linbro or Costar tissue culture plates. Stimulator lymphocytes receive 2000 R of gamma irradiation prior to culture and glioma cells receive 10,000 R of gamma irradiation. Responder lymphocytes are cultured at a density of $1-2 \times 10^6$ cells/well. Optimal glioma cell density has been found to be $0.5-1 \times 10^5$ cells/well for the cell lines studied thus far. Irradiated stimulator lymphocytes are most often used at a density of 1×10^6 cells/well. Cytotoxic cells are harvested after 7 days of culture, and their ability of lyse ^{51}Cr -labeled glioma targets is measured.

Glioma cells to be used as targets in lytic assays are incubated with $100 \mu\text{Ci } ^{51}\text{Cr}$ in 1 ml of culture medium for 1 hour at 37° . The cells are then washed extensively to remove any ^{51}Cr which was not incorporated into the cells. One tenth ml of cell suspension containing 10^5 glioma cells/ml is seeded into each well of a Falcon Microtest II culture plate. The cells are allowed to establish themselves for 18-24 hours. The culture medium is then aspirated from each well and replaced with 0.2 ml of lymphocyte suspension. Lymphocytes are incubated with glioma cells for 24 hours at 37° . At the end of this time, 0.1 ml of culture medium is withdrawn from each well, and the amount of ^{51}Cr contained in each sample is measured in a gamma counter. The total amount of ^{51}Cr which was contained in the glioma cells incubated with lymphocytes is calculated as $(e - c)/100 - c$ where e is the percentage of ^{51}Cr released from glioma cells incubated with lymphocytes and c is the percentage of ^{51}Cr released spontaneously from glioma cells incubated alone.

The chemotherapeutic studies are carried out by means of a similar microtiter system in which the diluted chemotherapeutic agents are under study (primarily the nitrosoureas BCNU and CCNU). The target cells from individual patients are exposed for varying periods of time to these therapeutic agents. Direct observations can be made of cell killing by means of phase microscopy and time-lapse microphotography as well as by cell counting similar to that done in the immunological techniques. A cytotoxic index can be established for each agent at each concentration. The cell killing at concentrations closest to those achievable in patients can then be compared to actual clinical responses seen in patients who are receiving such therapy.

A new in vitro chemotherapy technique enabling the testing of agents insoluble in aqueous media has been developed over the past year. Utilizing the aqueous insoluble drugs in the solid phase and the cell membrane itself as the solvent, this assay has made it possible to test a much wider range of potential antiglioma agents than was previously possible.

Correct histologic diagnosis and grading of human brain tumors is necessary to determine proper therapy. With the cooperation and histologic support of Drs. Jose Costa, Alan Rabson and Marious Valsamis of the NCI, Dr. Paul McKeever has established a diagnostic service for the Clinical Center which includes full time review of frozen and permanent section, special stains and electron microscopy of neurosurgical tissue by the neuropathologist. The service includes postmortem evaluation and review.

III. MAJOR FINDINGS:

Major findings over the past year can be divided into 1) cell biology/characterization; 2) chemotherapy; and 3) immunology categories.

1) Cell biology/characterization

In cell biology/characterization, the analysis of proteins released by glioma cells has been important. We have found that cells cultured from human astrocytomas biosynthesize and release a number of previously unknown proteins into the culture medium. Such proteins are of concern to the immunotherapist interested in directing humoral therapy to the tumor itself. Prototype mammalian gliomas also release a number of proteins. Studies are in progress to determine whether these proteins are true secretory products of astrocytes.

Changes in morphology and immunologic reactivity of the cells cultured from human brain tumors have occurred during explantation and culture. These changes are being studied by sequential light, electron and scanning electron microscopic evaluation. Biochemical evaluations include sequential assessment of glial fibrillary acidic protein, secreted proteins and collagen synthesis. Differentiating agents are being used to explore the

phenotypic range of glioma antigens. The differentiating agent dibutyryl cyclic AMP selectively inhibits the cellular release of one 47,000 dalton protein and simultaneously causes cell process formation in a human astrocytoma. This protein is being further characterized to determine its similarity to glial fibrillary acidic protein.

Another finding is that of junctional complexes (very possible gap junctions) between glioma cells in culture. This is an important finding because it confirms the glial origin of the cells grown in culture.

2) Chemotherapy

The new solid phase chemotherapy assay has shown that it is possible to test aqueous insoluble chemotherapy agents in vitro and that two drugs, AZQ and rapamycin may be effective antiglioma agents. AZQ is sufficiently promising that, with Phase I testing completely, a protocol for glioma patients is being instituted. In contrast, PCNU, a nitrosourea derivative has been found ineffective and will not be tested further. Spirohydantoin (chemically related to phenytoin) has been shown to have a delayed antiglioma effect (at 1 week) and in this respect confirms ongoing work on phenytoin itself.

The ability to correlate in vitro chemotherapy data with clinical response has been demonstrated in a series of 14 patients (manuscript accepted by Cancer). This is important because the in vitro microcytotoxicity methods must have such a correlation if they are to be useful.

Factors determining a cell's response or non-response to the chemotherapy agent BCNU have also been studied. The possibility that differences in DNA methylation repair from glioma cell line to glioma cell line has been explored with Dr. Rufus Day of the National Cancer Institute. Only one of several lines so tested has been found to have a positive correlation of inability to repair DNA methylation defects induced by BCNU and "positive" response in the microcytotoxicity assay. Other, likely cytoplasmic factors, appear important in the other lines. This is an important finding because it suggests new means by which to modify glioma cell sensitivity to nitrosoureas. In the light of this finding, the fact (also found this year) that cysteine (by virtue of its -SH content) can inhibit BCNU's cytotoxicity is all the more exciting since it is direct evidence of a cytoplasmic influence on an alkylating agent's cytotoxic action.

3) Immunology

In the humoral immunology assays further studies have continued to indicate high specificity for autologous patient glioma cytotoxicity testing. Not only is there a highly specific patient immunological response to gliomas, but, very importantly, the incidence of positive response is highest in patients with lower grade (Grade I-II astrocytomas), rather than the more

malignant Grade III-IV tumors, suggesting that the more malignant tumors escape immune detection and destruction. A further correlation is that a positive humoral antibody microcytotoxicity response can be correlated with longer survival times. The immune adherence assay, however, does not correlate with clinical survival. Finally, the immune adherence and microcytotoxicity assays have been determined to depend on different immunoglobulin classes, so that the mechanisms of tumor immune defense and lysis can be approached more effectively.

The finding that glioma cells release proteins into the extracellular medium has been important as well because it has provided new opportunities for the attempts at glioma antigen isolation now underway.

With respect to the cellular immunology, with the chromium assay, we have found that lymphocytes from three patients with malignant astrocytomas had little or no ability to lyse either autologous or allogeneic glioma cells when assayed for 24 hours at lymphocyte:target ratios as high as 100:1. One patient was tested both before and after surgery; two others were studied after surgery only. Thus these patients, none of whom had had immunotherapy, appear to have made little or no cell-mediated cytotoxic immune response to their tumors. The ability of these patients' lymphocytes to respond to the mitogens concanavalin A and phytohemagglutinin indicated that their T cell function in general was not grossly depressed. Freshly isolated lymphocytes from a number of normal volunteers have also been found not to lyse glioma cells in 24 hour ⁵¹Cr release assays. Thus glioma cells do not appear to be highly sensitive to NK-mediated lysis.

Freshly isolated peripheral blood lymphocytes from the three glioma patients studied thus far were not found to cause significant lysis of either autologous or allogeneic glioma cells. Nevertheless, lymphocytes from these patients could respond to the T cell mitogens Con A and PHa as well as to allogeneic lymphocytes in mixed lymphocyte cultures. Thus these patients seemed not to have made a detectable cytotoxic immune response to their own tumor although at least several in vitro assays of T cell function in these patients were grossly normal. Freshly isolated peripheral blood lymphocytes from normal volunteers have also been found not to cause significant lysis of glioma cells suggesting that glioma cells are not highly sensitive to the lytic effects of natural killer cells.

Incubation of lymphocytes with allogeneic glioma cells in mixed lymphocyte-tumor cultures leads to the generation of little or no cytotoxic activity. Thus glioma cells by themselves seem to be incapable of eliciting strong cytotoxic lymphocyte responses, perhaps explaining, at least in part, the lack of cytotoxic lymphocytes in the peripheral blood of glioma patients. Cytotoxic lymphocytes capable of causing specific lysis of allogeneic glioma cells can be generated if third-party

irradiated stimulator lymphocytes are added to cultures containing responder lymphocytes and glioma cells. This finding suggests the possibility that glioma cells may be deficient in their ability to stimulate helper T cells which facilitate the proliferation and differentiation of cytotoxic precursors into mature cytotoxic lymphocytes.

The work on the generation of anti-glioma cytotoxic lymphocytes and their interaction with glioma targets is complemented by studies in a murine model on the basic mechanisms by which immune lymphocytes interact with and lyse tumor cells. These studies require populations of cytolytic lymphocytes having very high lytic activity. Such high lytic activity cannot currently be attained in the antiglioma system described above nor indeed in any other human tumor system, and thus the use of a mouse model is necessitated. This model, which employs lymphoid tumor cells as targets, has previously been characterized extensively. Our current studies are focused on delineating molecules of potential importance in the generation of cytolytic T lymphocytes or in their lytic interaction with tumor target cells. CTL are endogenously labeled with ³⁵S-methionine during their differentiation in mixed lymphocyte cultures. Labeled cells are hypotonically lysed and then fractionated by differential centrifugation. Labeled proteins in the various subcellular fractions are examined by SDS-polyacrylamide gel electrophoresis. In initial experiments we have compared the plasma membrane proteins from a variety of lymphocyte populations. This has led to the discovery of a protein which we have called T11. This protein which has a molecular weight of 11,000 daltons, is a major component of the plasma membrane of T lymphocyte blasts activated in mixed lymphocyte culture or by concanavalin A but not T blasts activated by phytohemagglutinin (PHA). Interestingly, T blasts activated in mixed lymphocyte culture or by Con A but not T blasts activated by PHA possess cytolytic activity. T11 is virtually absent from the plasma membranes of normal splenic lymphocytes, B lymphocytes activated by E. coli lipopolysaccharide, and several lymphoid tumors. Attempts to further delineate whether T11 is on cytolytic T lymphocytes or on helper T lymphocytes are in progress. Likewise, we will attempt to purify T11 and to produce an antibody against it. This antiserum will be used to study the possible function of T11 in the generation and/or action of cytolytic T lymphocytes.

PROPOSED COURSE:

The past year has seen the establishment at the NIH of the brain tumor program initiated in the SNB last year. The integration of a new group of scientific experts into the program has been achieved. The time-consuming task of establishing totally new laboratory facilities has been largely completed and the intensification of both the in depth and extended studies of glioma cell biology, chemotherapy/growth modulation testing, and humoral and cellular immunology are now underway.

For the area of glioma cell biology and characterization, major goals include: improvement of a self-contained glial fibrillary acidic protein assay, glioma surface membrane analysis using scanning microscopy and x-ray spectrometry techniques, junctional and immunologic properties of the proteins secreted by gliomas. With respect to characterization of protein synthesis and release, comparison of glioma cells with myeloma and lymphoma models of multichained protein production will be pursued. Growth factor assays as developed for sarcoma cells will be applied to the glioma cells. Comparison of the properties of the gliomas induced in nonhuman primates by human JC virus will also be pursued.

In the chemotherapy/ growth regulation studies testing of new agents in both the solid phase and aqueous microcytotoxicity assays will be pursued. The positive results with AZQ testing will be put into a clinical trial through a new protocol. Modification of "response" or non-response by tumor cells to chemotherapy agents by growth factors and other biological growth and/or differentiation control agents will be pursued. Another major continuing effort will be the further determination of the precise predictive value of selective responsiveness and individualized, customized therapy in a larger population of patients. A prospective trial will be organized to devise or alter therapeutic plans based on in vitro responsiveness to given chemotherapeutic agents.

For the immunology studies, the newly initiated immunotherapy study protocol will be a high priority. Several subgoals will require strong effort in the coming year. These include a) enhancement of tumor cell growth in tissue culture to provide adequate cell numbers for frequent immunotherapy; b) continued dissection of both the cellular and humoral aspects of the host response as well as the specificity of the antigenic properties responsible for eliciting the host response; c) increased baseline immune studies in protocol patients; and d) further toxicity/safety studies in nonhuman primates and rabbits.

In the area of humoral immunology dissection of the humoral response (antibody classes, antigen types) will continue. It is apparent that immunoadherence and cytotoxicity are two significantly different aspects of humoral responsiveness and need to be studied in greater detail. Detailed absorption studies are necessary to determine the specificity of the reactivity. Antigen purification will be crucial to the elucidation of basic immune response and also a key to the SNB programs of active immunotherapy. Target cell factors in immune lysis will also be studied in detail in the coming year in hopes that these may yield ways of improving the cytotoxic immune response in patients.

For cellular immunology, studies on the generation of lymphocytes cytotoxic to glioma cells are still at a very early stage. Much further work is needed to define the identity and specificity of the cytotoxic effector cell produced in these experiments as well as the identities and specificities of the cells required for its generation. While much of the preliminary exploratory work will continue to be carried out in the allogeneic system, efforts to generate glioma-specific cytotoxic lymphocytes using autologous combinations of lymphocytes and glioma cells will be intensified as rapidly as the availability of reagents permits. If cytotoxic lymphocytes specific for glioma antigens can be generated in autologous systems, collaborative studies with Dr. Eugene Quindlen will be initiated to examine the ability of humoral antibody to affect the generation and action of glioma-specific cytotoxic lymphocytes. Likewise, whether the action of such cytotoxic lymphocytes is HL-A restricted will be studied. We will attempt to modify glioma cells so as to make them capable of directly eliciting cytotoxic cells in the absence of added stimulator lymphocytes.

Attempts will be made to modify glioma cells by enzyme treatment, by haptentation, or by introducing new macromolecules into the glioma cell membrane via fusion with liposomes, and the ability of the modified cells to directly elicit a cytotoxic immune response in vitro will be examined. These types of studies may in turn lead to the development of more effective programs of immunotherapy to enhance the patient's cellular immunity to his own tumor. Also we will initiate studies to determine whether glioma cells secrete proteins or other substances which possess immunosuppressive properties or are otherwise capable of impeding the attack of cytotoxic lymphocytes on glioma cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02368-02 SN
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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biological and Immunological Factors in Peripheral Nerve
Regeneration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry H. Smith	Medical Officer	SNB	NINCDS
	Paul L. Kornblith	Chief	SNB	NINCDS

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Surgical Neurology Branch

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.35

PROFESSIONAL:

0.10

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS

☒ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In addition, a protocol to study factors in failed human peripheral nerve regeneration has now been set up and approved by the NINCDS Human Research Subpanel. This is Protocol No. 80-N-06. The first patient candidates are now being reviewed to determine their acceptability for this protocol.

The cellular, biological and immunological factors in peripheral nerve regeneration are being studied in a rat vein-graft model. The vein graft serves as a chamber into which various biological agents such as collagen or "trophic" factor such as nerve growth factor as well as specific cell types grown in tissue culture can be added to study their effects on axonal regeneration. Quantitative light and electron microscopic measures are being utilized to analyze the effects. To date, human tumor cells in the vein-graft have enhanced regeneration whereas microcrystalline collagen has inhibited the process. The influence of fetal dorsal root ganglion cells, cerebellar cells, cortical cells, and fibroblasts are currently under study.

PROJECT DESCRIPTION:

I. Objectives: A study of the cellular and macromolecular factors influencing the success or failure of regeneration of axons after peripheral nerve injury in both a rat experimental model as well as in patients with peripheral nerve injuries.

II. MATERIALS AND METHODS:

A. Nerve injury and graft: The left or right sciatic nerve in either an Osborne-Weber or caesarian-delivered Fisher rat is transected sharply. Thereafter it is either 1) left alone for control; 2) reapproximated directly via a nerve graft with 9-0 nylon; 3) repaired with a 5 mm segment of vena cava obtained from a second animal of either the same or the other rat strain.

B. Vein graft placement: Under surgical microscopic control, the 5 mm segment of donor vein is sewn to the perineurium of the proximal and distal nerve ends with a total of four 8-0 nylon sutures. Care is taken to assure minimum tension.

C. Cell preparation for placement in graft: Rat or human cells are obtained from either the tissue culture stock lines in our laboratory or grown from new fetal cell cultures of the central nervous system as well as fibroblastic elements. Cultures from the different tissues as well as regions of the brain are quite distinct morphologically in tissue culture and maintain these characteristics over time. At the time for injection into the nerve graft, they are removed from the flask surface, centrifuged to provide a concentrated pellet and injected via a #27 needle into the graft.

D. Macromolecular factors: Microcrystalline collagen (nonantigenic) is placed within the vein graft prior to suturing under microscopic control to fill the vein graft cavity.

E. Follow-up periods: Animals so treated (see A-D above) are then followed for periods ranging from 0 time to 6 months prior to histologic study.

F. Histologic and ultrastructural examinations: All nerves and/or grafts are removed for study and cut into 1 mm segments to allow for precise reconstruction of the nerve. Fixation is accomplished with glutaraldehyde with standard Epon embedding. Thick sections are then cut for light microscope evaluation and thin sections are prepared for electron microscopy (uranyl acetate staining). Quantitation of numbers regenerating myelinated and unmyelinated axons is done by both light and EM methods.

G. Human peripheral nerve tissue and/or scans or neuromas will be obtained under Protocol No. 80-N-06. Light and electron microscopic examination of the area of failed peripheral nerve regeneration are carried out when possible in accordance with

optimal medical and surgical care standards. Analysis includes clinical data (nerve conduction, evoked potentials, functional recovery) as well as histologic and ultrastructural examinations as per "F" above.

III. MAJOR FINDINGS: The major findings to date include:

A. Long-term regeneration studies (6-9 months) are now in the process of being analyzed. The inhibitory effects on regeneration of heterologous and/or allogeneic grafts have been confirmed. Some regeneration does occur by six months post-grafting in such cases of immunological mismatching but it is less than in controls as would be expected.

B. The influence of human glioma cells (Line L.M. injected into the graft continues to be of interest because of a potential promoting effect on myelinated axonal regeneration. At longer times (up to 6 months) it is not clear what the residual influences of the L.M. cells may be. The "foreign" cells disappear with the first two months. The "central" or "peripheral" origin of the myelin formed in these cases is currently under study.

C. Microcrystalline collagen, placed in the graft, was previously reported to inhibit the regeneration process over periods up to 8 weeks. Now 6-8 month studies have been completed and that data is being analyzed. Even at the long periods studied, there are retained pockets of crystalline collagen. No axonal regeneration occurs in these areas, confirming the earlier studies, but adding the further caution of long-term deleterious affects of such collagen on nerve regeneration.

D. The study of influence of various fetal rat cell lines is proceeding with vein grafts now taken from animals at 1 week to 6 months post-grafting. None of these lines, including the fibroblasts, has proven as yet to have either a major growth promoting or inhibitory effect on the regeneration. The time over which such cells survive in the graft is being carefully evaluated.

E. A patient protocol for the study of causes of failed human peripheral nerve regeneration has been instituted and two patients' neuromas are now under intensive study.

IV. SIGNIFICANCE:

Repair of damage to the nervous system is a major problem for basic and clinical neuroscience and the peripheral nerve injury and repair model under study here represent an approach to the study of this process. The project coincides with Institute interests as exemplified by the Stroke, Trauma and Tumor Program. Improving nerve tissue regeneration is one of the highest priority areas in neurobiology today.

V. PROPOSED COURSE:

The rat model studies will continue to be studied utilizing both light and electron microscopic techniques, including quantitative image analysis to determine the effects of the local environment on the regenerating axons. In the rat model analysis of the influence of fetal dorsal root ganglion cells, cerebellar cells, cortical cells and fibroblasts will be completed. Other graft matrices including various cell growth promoting and inhibiting factors will also be studied. The implementation of a clinical protocol concerned with the causes of failed peripheral nerve regeneration will provide human material for tissue culture as well as morphological study. Optimal clinical care will be provided as a part of this protocol as well.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01047-18 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Radionuclide Ventriculography and Cisternography Previous Title: Isotope Ventriculography and Isotope Cisternography		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	G. Di Chiro	Chief, Neuroradiology and Computed Tomography Section
OTHER:	G.S. Johnston A.E. Jones R.A. Brooks	Chief, Nuclear Medicine Dept. Assistant Chief Senior Staff Fellow
		SN NINCDS NM CC NM CC SN NINCDS
COOPERATING UNITS (if any) Nuclear Medicine, Clinical Center, NIH		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.083	PROFESSIONAL: 0.083	OTHER: 0.0
CHECK APPROPRIATE BOX(ES)		
<input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>Radionuclide <u>ventriculography</u> and cisternography are diagnostic tools permitting the morphologic and dynamic study of the cerebrospinal fluid pathways more accurately than has ever been possible with any other diagnostic test.</p> <p>The adjunction of <u>positron emission computed tomography</u> to our diagnostic armamentarium should improve significantly the information content of our radionuclide ventriculograms and cisternograms.</p>		

Project Description:

Objectives: A gamma-emitting isotope injected within the cerebrospinal fluid pathways will permit in subsequent head scans the pictorial outline of the ventricular system (isotope or radionuclide ventriculography) and of the subarachnoid intracranial spaces (isotope or radionuclide cisternography). Information about the anatomical status of the cerebrospinal fluid cavities, and, by multiple serial scans, of the normal and abnormal dynamics of the cerebrospinal fluid itself will be obtained. The spinal CSF spaces may also be evaluated.

Methods Employed: The radionuclide cisternography and ventriculography procedures are now well established.

Recently we have devoted particular attention to one aspect of the CSF flow, i.e., its descent to the spinal subarachnoid space.

Major Findings: We have initiated positron emission computed tomography (PECT) of the CSF cavities after intraventricular (IV ventricle) introduction of a positron emitter ($^{68}\text{GaEDTA}$) in primates.

Significance to Bio-Medical Research and the Program of the Institute: Legions of authors are studying this remarkable fluid (CSF) which still remains in many respects uncomprehended since Cotugno first described it in 1764. In particular we now have a diagnostic tool to gather information about the "terra incognita" which is represented by the basal and convexity subarachnoid pathways, as well as the spinal CSF compartment. In this area, the CSF spinal descent studies should enable us to determine what is the importance of the spinal CSF route of flow as an alternative pathway of resorption. The observations of the spinal descent pattern of the CSF have also heuristic significance in regard to a possible analysis of metabolites and drugs distribution through the CSF from the endocranial cavity to the easily accessible spinal theca.

Proposed Course of Project: Further information about the normal and abnormal cerebrospinal fluid cavities, and the normal and pathologic flow of CSF will be gathered by the techniques of radionuclide cisternography and ventriculography. The adjunction of the capabilities for PECT (an ORTEC-ECAT, PECT device has been installed and is operational at the NIH Clinical Center) permits significant refinements in the techniques of radionuclide cisternography and ventriculography. In particular, the use of radiopharmaceuticals tagged with positron emitters (e.g., chelating substances labeled with ^{68}Ga) allows for a better demonstration of the tagged CSF in the deep CSF cavities. This improved demonstration is possible through the tomographic display of images representing axial transverse slices. The problem of the superimposition of the radioactivity in the superficial tissues, so disturbing in the interpretation of conventional radionuclide CSF scinti-photographic studies, is practically eliminated. The NIH Neuro-PET, which is presently being built will offer soon further resolution capabilities of the CSF cavities with PECT.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01195-16 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Radiographic and Radioisotopic Angiography of the Spinal Cord</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	G. Di Chiro	Chief, Neuroradiology and Computed Tomography Section
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		DR CC
	J. R. Herdt	Deputy Chief
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	P. L. Kornblith	Chief
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	E. Quindlen	Senior Staff Fellow
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	G. S. Johnston	Chief
		NM CC
	A. E. Jones	Assistant Chief
		NM CC
COOPERATING UNITS (if any) Greater S.E. Community Hosp., Wash., D.C.: Hosp. of the Univ. of PA, Philadelphia, PA: Medical Examiner's Office, Dept. of Public Health, Philadelphia, PA: Diagnostic Radiology and Nuclear Medicine Depts., Clinical Center, NIH.		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.083	PROFESSIONAL: 0.083	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Selective arteriography</u> (radiographic) of the spinal cord is a diagnostic technique which has proven to be very informative in cases of arteriovenous malformation, tumor, obstructive vascular disease, trauma, and postradiation damage of the spinal cord. </p> <p> <u>Radioisotope angiography of the spinal cord</u> offers distinct advantages as a screening method, and in certain types of intraspinal pathology may give information not available by any other diagnostic test. </p> <p> <u>Preliminary experience with computed tomography of the spine after injection of contrast medium</u> indicates that this methodology is useful in the evaluation of certain vascular lesions of the spinal cord. </p>		

Project Description:

Objectives: The introduction of cerebral angiography (1927) has markedly increased our knowledge of the vascular pathology of the brain. The vascular pathology of the spinal cord, on the other hand, still remains a largely unexplored area.

Since 1964 we have been carrying out angiographic studies of the spinal cord and developed this technique into a reliable diagnostic tool. Selective injection of the contrast medium has made the difference between an occasional demonstration, and the consistent visualization of the spinal cord vasculature.

The usefulness of selective arteriography in cases of spinal cord arteriovenous malformations is now well established. We are continuing to use this technique to:

1) Learn more about the pathophysiology of the spinal cord arteriovenous malformations so that a better treatment of these important and frequent lesions may be developed.

2) Evaluate how useful spinal cord angiography is in cases of spinal cord tumors.

3) Establish whether or not this technique can be of diagnostic value in the study of obstructive spinal cord vascular disease.

4) Assess the usefulness of this technique in intervertebral disc pathology.

5) Evaluate the diagnostic possibilities of this procedure in post-traumatic spinal cord injury with or without vertebral fractures.

6) Establish the value and limits of newly introduced radioisotopic angiography of the spinal cord.

7) Explore the possible emergency therapeutic means which could be employed to treat and cure, or at least minimize the effects of the dreadful postangiographic cord complications.

8) Acquire new information regarding the fine vasculature of the human spinal cord, with particular emphasis on the intrinsic vessels (sulcal and central arteries and other perforating or penetrating branches).

This goal is accomplished by post-mortem microangiographic techniques in cadavers of all age groups. We are paying particular attention to cords of aged adults.

Methods Employed: Selective arteriograms with modern catheter techniques are carried out in patients in whom spinal cord vascular or tumoral lesions are suspected. Rapid serialograms, subtraction and magnification are used to better visualize the injected vessels.

For the technique of radiosotope angiography of the spinal cord a bolus of 15 mCi of ^{99m}Tc human serum albumin or $^{99m}\text{TcO}_4^-$ is injected in a left antecubital vein. Immediately afterwards, scintiphotographic rapid flow studies of the various segments of the spine are obtained with a scintillation camera. Our scintiphotographic data have been significantly ameliorated by a computer assisted analysis and reconstruction of images, as well as by isometric contour computer display of the data.

For the technique of computed tomographic angiography of the spinal cord we use a computed tomography (CT) body scanner and we carry out timed serial tomograms of the area of interest of the spine after the intravenous introduction of a bolus of angiographic contrast medium.

For the post-mortem studies of the vessels of the human spinal cords, (aged adults) we have used our previously developed microangiographic techniques.

Based on the observation made elsewhere, that in two patients who died soon after aortography with spinal cord complications, the iodine content in the CSF was enormously increased, we have been attempting an emergency therapeutic method consisting of flushing out (lavage) the "iodine contaminated" CSF.

Major Findings: We have continued to accumulate experience in the areas of:

- 1) Selective arteriography in vascular malformations of the spinal cord.
- 2) Selective arteriography in cases of herniation of thoracic discs.
- 3) Post-mortem microangiographic evaluation of the aged human cord.
- 4) CSF lavage in patients who develop symptoms and signs of cord involvement after abdominal aortography or other types of arteriographic studies.

Significance to Bio-Medical Research and the Program of the Institute: Radiographic and radioisotopic angiography of the spinal cord are increasing our understanding of the large group of conditions in which vascular lesions of the cord represent the basic pathologic element.

Proposed Course of Project: Post-mortem microangiography of the aged adults' cords should offer new insights on such conditions as obstructive vascular disease of the cord due to arteriosclerosis and cervical spondylosis, and possibly on degenerative and demyelinating cord diseases.

We are "watching" for possible further technical developments of the technique of selective arteriography of the spinal cord. We are considering initiating the use of angiotomography for a better visualization of the smaller vessels, possibly the intrinsic arteries and veins of the cord.

Improved x-ray vascular contrast media will also enhance the diagnostic possibilities of spinal cord angiography. We are following very closely the recent developments in the area of polymeric, ion-balanced and non-ionic iodinated x-ray contrast media.

Radioisotope angiography of the spinal cord is a method which we have been using as a screening and follow-up procedure.

Computed tomographic (transmission) angiography of the spine and spinal cord represents one of the areas in which we will concentrate a great deal of interest.

We have some expectation that positron emission computed tomography particularly with the use of the high resolution Neuro-PET (designed in our Section), will allow us to study blood flow and metabolism (glucose metabolic rate) of the cord in a non invasive-fashion.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01654-13 SN																				
PERIOD COVERED October 1, 1979 to September 30, 1980																						
TITLE OF PROJECT (80 characters or less) Experimental Spinal Cord Angiography																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">G. Di Chiro</td> <td style="width: 30%;">Chief, Neuroradiology and Computed Tomography Section</td> <td style="width: 10%;">SN</td> <td style="width: 5%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>E. Quindlen</td> <td>Senior Staff Fellow</td> <td>SN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>P. L. Kornblith</td> <td>Chief</td> <td>SN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>B. H. Smith</td> <td>Senior Staff Physician</td> <td>SN</td> <td>NINCDS</td> </tr> </table>			PI:	G. Di Chiro	Chief, Neuroradiology and Computed Tomography Section	SN	NINCDS	OTHER:	E. Quindlen	Senior Staff Fellow	SN	NINCDS		P. L. Kornblith	Chief	SN	NINCDS		B. H. Smith	Senior Staff Physician	SN	NINCDS
PI:	G. Di Chiro	Chief, Neuroradiology and Computed Tomography Section	SN	NINCDS																		
OTHER:	E. Quindlen	Senior Staff Fellow	SN	NINCDS																		
	P. L. Kornblith	Chief	SN	NINCDS																		
	B. H. Smith	Senior Staff Physician	SN	NINCDS																		
COOPERATING UNITS (if any) J. Fein, Department of Neurological Surgery, Albert Einstein College of Medicine, Bronx, NY, formerly of Armed Forces Radiobiology Research Institute, Bethesda, MD; K. Earle, Chairman, American Registry of Pathologists, Washington, D.C.																						
LAB/BRANCH Surgical Neurology Branch																						
SECTION Neuroradiology and Computed Tomography Section																						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																						
TOTAL MANYEARS: 0.083	PROFESSIONAL: 0.083	OTHER: 0.0																				
CHECK APPROPRIATE BOX(ES)																						
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER																						
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords) <u>Experimental spinal cord angiography</u> in the rhesus monkey is increasing our understanding of the blood supply of the spinal cord both in physiological and pathological conditions. Recently this technique has been used as a basis for attempts at experimental surgical revascularization of the cord in primates.																						

Project Description:

Objectives: The clinical value of the NIH developed technique of selective arteriography in the management of arteriovenous malformations and tumors (in particular hemangioblastomas) of the spinal cord is now well established.

In order to expand the clinical applications of arteriography of the spinal cord we are working with experimental angiographic and microangiographic models in primates.

Previously, we have concentrated our attention on the area of experimental obstructive vascular disease of the spinal cord in the rhesus monkey. More recently much of our experimental investigation has dealt with a catastrophic iatrogenic pathological condition, postradiation myelomalacia (myelitis), which occurs more frequently than is generally realized. In this area we are trying to establish whether the basic pathological lesion of this dreadful complication is primarily neurogenic or vascular.

In this fiscal year we have initiated the use of spinal cord arteriography in primates as an indispensable prerequisite to attempts at revascularization of the thoraco-lumbar spinal cord.

Methods Employed: Preradiation angiographic studies (selective technique) of the thoracolumbar segment of the spinal cord are carried out in young, healthy rhesus monkeys. Soon after, selective irradiation of the thoracolumbar cord using a LINAC accelerator is initiated. Total dosage and modalities of delivery are chosen to approximate the radiation protocol which most often seems to cause myelomalacia in human patients. At the end of the radiation, the monkeys are kept under careful observation for periods of many months. Neurological testing of the lower limbs is performed twice a week. If and when the monkeys show signs of developing or established paraplegia, repeat selective arteriography of the irradiated segment is carried out. Following this, the animals are perfused for microangiography of the spinal cord and then sacrificed. The cord is studied by gross observation, microangiography, routine histology and special myelin stains. Careful gross and histological analysis of the neighboring aortic segment, its branches, and the pertinent radiculomedullary arteries is also carried out.

For the cord revascularization project we carry out presurgical spinal cord arteriography in the rhesus. In this primate the arteria radiculomedullaris magna (artery of Adamkiewicz) most frequently originates from the second left lumbar artery. After angiography has provided us the basic information on the cord vascularization, we perform surgery to anastomose one of the last intercostal arteries (XI, XII) with the anterior spinal artery above the artery of Adamkiewicz anastomosis. For this purpose a transectomy and partial resection of one vertebral body is necessary. The anastomosis is end-to-side with the help of the surgical microscope. If and when we will be able to accomplish successful anastomoses, the artery of Adamkiewicz will be ligated. As we have proven in previous studies (Fried, L.C., Di Chiro, G. and Doppman, J.L. Ligation of major thoraco-lumbar spinal cord arteries in monkeys. J. Neurosurg. 31:608-614, 1969), ligation of this

artery in normal monkeys always causes paraplegia. In the "revascularized" monkey, this should not be the case because the thoraco-lumbar cord, conus and filum terminale would be fed by the anastomotic intercostal artery.

Major Findings: We are on the course of evaluating the pathological changes of the spinal cord from monkeys in which we successfully induced postradiation paraplegia (myelopathy). Our efforts with experimental cord revascularization in primates have just begun.

Significance to Bio-Medical Research and the Program of the Institute: We should be able to shed some light on the pathogenesis of the postradiation myelitis. This is not a rare complication in human patients (over 500 cases have been reported in literature). The implications of a successful revascularization of the cord for the many human patients suffering from ischemic cord disease are obvious.

Proposed Course of Project: Appraisal of the postradiation data which we have already collected as well as new data in other irradiated animals now under observation. We will attempt to study (by angiography and micro-angiography) human patients (or human specimens) with postradiation spinal cord damage. Our effort with the cord revascularization project will proceed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02073-07 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) New Title: Computed Tomography (Transmission) Previous Title: Computer Assisted Tomography (Transmission Computed Tomography)		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: G. Di Chiro R.A. Brooks K.G. Rieth V.J. Sank OTHER: P.L. Kornblith B.H. Smith E. Quindlen J.R. Herdt G.S. Johnston A.E. Jones Continued on Page 2	Chief, Neuroradiology and Computed Tomography Section Senior Staff Fellow Staff Radiologist Research Assistant Chief Senior Staff Physician Senior Staff Fellow Deputy Chief Chief Assistant Chief	SN NINCDS SN NINCDS DR CC SN NINCDS SN NINCDS SN NINCDS DR CC NM CC NM CC
COOPERATING UNITS (if any) Neurosurg. Service, Dept. of Psychiatry and Neurology, WRAMC Wash., DC; Physics Dept., Tufts Univ., Medford, MA; Cyclotron Lab., Harvard Univ., Cambridge, MA; Diagnostic Radiology, Nuclear Medicine Depts., CC; Pediatric Oncology Branch, DCT, NCI; Infectious Diseases Branch, Laboratory of LAB/BRANCH Neuropathology and Neuroanatomical Sciences, IRP, NINCDS, NIH. Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.58	PROFESSIONAL: 1.58	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Computed Tomography</u> (CT) in its transmission and emission modalities, represents the main research area of the Neuroradiology and Computed Tomography Section. Ongoing clinical -animal/experimental research projects in transmission CT include studies of degenerative, demyelinating and atrophic processes of the brain, hydrocephalus, brain edema, postradiation cerebral necrosis, surgically correctable lesions in young patients affected by chronic epilepsy, diseases of the spine and the spinal cord, attempts at tissue characterization of normal and abnormal (e.g. tumoral) cerebral tissue, and an experimental glioma model in primates. Physics projects: improved dual-energy CT scanning using both a split-detector and a dual kVp method; analysis of aliasing effects and developments of methods for their elimination; phantom studies for the evaluation of artifacts and calibration of CT machines. Feasibility tests to build a new type of CT device which will use <u>protons</u> are under way.		

Names, Laboratory and Institute Affiliation, etc. (Cont'd)

OTHER: J.L. Sever	Chief	ID NINCDS
W.T. London	Chief, Experimental Pathology Section	ID NINCDS
I. Klatzo	Chief	LNNS NINCDS

Cooperating Units (Cont'd)

Infectious Diseases Branch, Laboratory of Neuropathology and Neuro-anatomical Sciences, IRP, NINCDS, NIH.

Project Description:

Objectives: To advance the clinical applications of CT. Attention is being devoted to trying to improve the resolution of the CT devices. Advanced quantitative assessment of the attenuation values (profiles, regions of interest) are used in an attempt to improve the diagnostic specificity of CT; the goal is to enhance our capability of distinguishing between different types of lesions which present with similar qualitative findings. Differentiation of the various tissues' chemical components (tomochemistry) through dual-energy scanning or, possibly in the future, by means of proton CT, is a promising line of our research. An experimental glioma model in primates offers great hopes for improvement of our knowledge of the optimal parameters for CT scanning of the brain and also for enhancing our ability to differentiate between the various glioma types.

Methods Employed: Clinical CT neuro-scanning is now a standard diagnostic procedure. Groups of patients with various disease conditions are studied by CT of the brain and/or spine-spinal cord.

A split detector of original design has been built to carry out simultaneous dual-energy scanning.

An experimental (virus induced) glioma model in primates has been developed and tested and is being used for a variety of purposes.

Basic experiments being carried out as a preliminary step to build the PROTO-Scanner, involve determination in various phantoms of the absorption coefficient of the proton beam produced by a cyclotron. The phantoms include organic materials (particularly organic solutions of various concentrations).

Major Findings: In the clinical area we have:

- 1) Carried out a study of the CT attenuation profiles in the periventricular regions in patients affected by a wide variety of pathological conditions in which periventricular areas of hypodensity are recognized (hydrocephalus, multiple sclerosis, the leukodystrophies, leukoencephalopathies related to dismetabolic processes or to mitochondrial abnormalities, leukomalacia in the area of the germinal matrix, progressive multifocal leukoencephalopathy, disseminated necrotizing leukoencephalopathy, subacute sclerosing panencephalitis, viral inflammatory processes, post-radiation necrosis of

the brain, and periventricular spreading of primary or secondary CNS neoplasm). Categorization and differentiation of the various CT profiles have been accomplished. Profile characteristics for particular conditions have been demonstrated. For instance, on the basis of the CT attenuation data, we are now able to discriminate between the periventricular hypodensity related to hydrocephalus (especially in its acute form) and the hypodensity connected with the various types of leukoencephalopathy. The concept of the "ventricular wall barrier," as identified by CT, has been introduced. Computed tomography permits the distinction between the intact and the disrupted (Impaired) barrier.

2) Dual-energy CT (Tomochemistry) - A significant amount of dual-energy CT data on patients has been accumulated. Our goal is to establish dual-energy CT "signature" of the various tissues. With this technique, the CT recognition of even minimal amounts of certain tissues (particularly fat) as well as electrolytic fluids (CSF) is greatly improved as compared to conventional CT. The split detector developed in our Section for the purpose of dual-energy scanning has been helpful in these studies.

3) A new and advanced computer program has been developed for measuring CSF volume (ventricular and subarachnoid cavities).

4) Our CT research on the spine and spinal cord has continued. In the last year emphasis has been put on the comparison between CT and radionuclide scanning of the spine in metastatic processes. Efforts at improving the CT resolution of the spinal cord and the spinal CSF have continued and further experience with our technique of computer assisted myelography (CAM) has been accumulated.

5) Observed interesting findings concerning postradiation necrosis of the brain. These findings may mimic brain tumors (recurrence or spread). Their recognition, therefore, is of capital importance.

6) Analyzed a large group of patients affected by chronic epilepsy to determine how frequently surgically correctable epileptogenic lesions can be detected solely by CT.

In the animal experimental area we have:

1) Completed a CT study on the edema in primates. Cryogenically-induced cerebral edema in the rhesus monkey was analyzed by serial CT scans in both axial and coronal planes. The onset, progression (peak at the fourth - fifth day) and resolution of the vasogenic cerebral edema have been assessed. An attempt was made to correlate the low CT attenuation values of the involved areas with the specific gravity of corresponding fresh edematous brain specimens.

2) In tandem with our clinical activity connected with the differentiation of the various types and stages of hydrocephalus, we have been performing experimental studies in primates with a variety of obstructive hydrocephalus models to evaluate timing of appearance and evolution of the periventricular hypodensity (thought to be related to the transependymal passage of CSF).

3) In tandem with our clinical activity on the spinal cord and spinal CSF we have carried out experiments in primates trying to develop better visualization of the cord and the spinal CSD through intravenous enhancement (intravenous CT myelography). From preliminary observations, it would appear that possibly there is an early relative enhancement of the cord followed by late relative enhancement of the CSF. By exploiting these features one could extract valuable additional information from CT of the spine after simple intravenous injection of contrast medium and, thus avoid the intrathecal administration.

4) Developed a model of experimental (virus-induced) glioma in primates. In the physics area the most important findings are:

- a) Algorithm improvements. Significant activity has been put into eliminating algorithmic artifacts from CT scans. This work has been highly productive, and in fact has been adopted by the leading CT manufacturer with a definite benefit to image quality.
- b) Development of a dual-energy Hounsfield tissue signature, and demonstration of its usefulness in patient diagnosis.
- c) Development of an offset detector method for the elimination of aliasing artifacts in fan-beam third-generation scanners.

Significance to Bio-Medical Research and the Program of the Institute:
The diagnostic abilities in the area of neuroradiological disease are fundamentally altered by the introduction of CT. The progress in this area is fast. Statements regarding the future significance of this methodology could be surpassed and rendered obsolete in a short time.

Proposed Course of Project: In the Neuroradiology and Computed Tomography Section, CT will be the main area of research for years to come. We will proceed with a multipronged approach: 1) clinical work on the brain, sella turcica-pituitary gland (microadenomas), spinal cord and eye; 2) experimental research on primates; 3) tomochemistry of the CNS; 4) theory (mathematics, physics); 5) planning and building a new type of CT device (using protons rather than x-rays).

A new journal, "JOURNAL OF COMPUTER ASSISTED TOMOGRAPHY", originates from this section (Eds.: DiChiro and Brooks).

Publications:

Jabbari, B., Di Chiro, G., and McCarty, J. O.: Mesial temporal sclerosis detected by computed tomography. J. Comput. Assist. Tomogr. 3:527-529, 1979.

Di Chiro, G., Brooks, R. A.: The 1979 Nobel Prize in Physiology or Medicine. Science 260:1060-1062, 1979.

Brooks, R. A., Talbert, A. J., Weiss, G. H.: The offset detector in computed tomography: Interleaving vs. separate processing.
IN: Raviv, J., Greenleaf, J. F., Herman, G. T. (Eds.) Computer Aided Tomography and Ultrasonics in Medicine. Amsterdam, New York, Oxford, Elsevier-North Holland Publishing Company, 1979, pp. 7-9.

Jabbari, B., Huott, A. D., Di Chiro, G., Martins, A. N., Youngblood, L.A., and Harper, M. G.: Surgically correctable lesions detected solely by computed tomography in adult onset, chronic epilepsy. Annals of Neurology 7:344-347, 1980.

Rieth, K. G., Fujiwara, J., Di Chiro, G., Klatzo, I., Brooks, R. A., O'Connor, C. M., Mitchell, L. G.: Serial measurements of CT attenuation and specific gravity in experimental cerebral edema. Radiology 135:343-348, 1980.

Morgenthaler, D. G., Brooks, R. A., Talbert, A. J.: Noise factor of polychromatic x-ray beam in computed tomography. Phys. Med. Biol. 25: 251-259, 1980.

Talbert, A. J., Brooks, R. A., Morgenthaler, D. G.: Optimum energies for dual energy computed tomography. Phys. Med. Biol. 25:261-269, 1980.

Rieth, K. G., Di Chiro, G., London, W. T., Sever, J. L., Houff, S. A., Kornblith, P. L., McKeever, P. E., Buonomo, C., Padgett, B. L. and Walker, D. L.: Experimental glioma in primates: A computed tomography model. J. Comput. Assist. Tomogr. 4:285-290, 1980.

Di Chiro, G.: Improvement in computed tomography spatial resolution. Proceedings of the International Symposium on Computerized Tomography, September 20-22, 1979. (In press)

Di Chiro, G., Eiben, R. M., Manz, H.J., Jacobs, I. B., and Schellinger, D.: A different (Type II) cerebral computed tomographic pattern in adrenoleukodystrophy. Radiology. (In press)

Brooks, R. A., Di Chiro, G., Keller, M. R.: Explanation of cerebral white-grey contrast in computed tomography. J. Comput. Assist. Tomogr. (In press)

Sheridan, W. T., Keller, M. R., Brooks, R. A.: Evaluation of edge artifacts in computed tomography. Med. Phys. (In press)

Keller, M. R., Kessler, R. M., Brooks, R. A., Kirkland, L. R.: Optimum imaging of iodinated contrast materials in computed tomography. Brit. J. Radiol. (In press)

Brooks, R. A., Sheridan, W. T., Keller, M. R., O'Connor, C. M., Mitchell, L. G.: Progress toward quantitative computed tomography. IEEE Trans. Nucl. Sci. (In press)

Weiss, G. H. Brooks, R. A.: Angular interpolation error in computed tomography. (submitted to Med. Phys.)

Names, Laboratory and Institute Affiliations, and Titles of Principal Investigators and All other Professional Personnel Engaged on the Project

Continued:

	G.S. Johnston	Chief	NM CC
	A.E. Jones	Assistant Chief	NM CC
	R.M. Kessler	Staff Physician	NM CC
	R.G. Blasberg	Medical Officer	LCP NCI
	A.P. Wolf	Senior Chemist	Brookhaven
OTHER:	L. Sokoloff	Chief	LCM NIMH
	D.E. Kuhl		UCLA
	M.E. Phelps		UCLA

Project Description:

Objectives: Recent new developments have made a significant difference in the practical and clinical application of PECT. The two most important of these developments are: 1) efficient PECT devices (scanners) have been developed and some are commercially available, and 2) the original Sokoloff's autoradiographic technique for determining local glucose metabolic rate in experimental animals has been converted into a PECT method for living human subjects (see below).

Methods Employed: The radioactivity originating from a positron emitting radionuclide, which generally is introduced by the intravenous route, is detected plane by plane (tomography) with axial-transverse (horizontal) or coronal incidence, and the images of this distribution in slices or cuts through the body area of interest are produced, displayed and recorded in a variety of fashions. The most interesting radionuclide is at present ^{18}F 2-deoxyglucose (^{18}F FDG). The application of this tracer is a direct derivation of the original, NIH-developed, Sokoloff's autoradiographic technique in experimental animals. In living human patients, it is now possible with ^{18}F (110 minute half-life) tagged 2-deoxyglucose to obtain pictorial data (axial transverse and coronal images) as well as quantitation (mg/min/100 gm of brain substance) of the local cerebral glucose metabolic rate. Other positron emitting radiopharmaceuticals of interest are ^{68}Ga chelated tracers (^{68}Ga is generator produced and has a 68 minute half-life); the ^{68}Ga tracers would be particularly interesting for the analysis of the CSF circulation (PECT cisternography). The use of tracers tagged with the short-lived ^{11}C , ^{13}N and ^{15}O will require a cyclotron on the NIH premises.

Major Findings:

1) The protocol project " ^{18}F -2-Fluoro-2-deoxy-D-glucose (FDG) Positron Emission Computed Tomography (PECT) in Typing of Cerebral Gliomas" has been initiated with the study by PECT of our first patients with cerebral glioma. This has been possible through the installation at the NIH of a commercial (ORTEC - ECAT) PECT scanner and the procurement from the Brookhaven National Laboratory of the FDG. (This procurement represents an interim arrangement until the NIH production of FDG is feasible.)

2) Two new protocols are in the approval stage: ¹⁸F-2-Fluoro-2-deoxy-D-glucose (FDG) Positron Emission Computed Tomography (PECT) in Epilepsy" and "Positron Emission Tomography to Determine Regional Cerebral Metabolism at Rest and During Cognitive or Pharmacological Stimulation in Patients with Alzheimer's Disease, Huntington's Disease and Those at Risk for Huntington's Disease."

3) The actual construction of the new, high resolution, NIH-built PECT-scanner (the Neuro-PET) is now underway.

4) A great deal of activity has been spent to solve the problems connected with the choice, purchase, and location/installation planning for the cyclotron at the NIH.

Significance to Bio-Medical Research and the Program of the Institute:
Following are the research projects which are considered significant to the program of the NINCDS and which we subdivide into two groups, (1) those using relatively long-lived radionuclides and (2) those employing short-lived radionuclides.

Group 1

A) Regional cerebral glucose consumption using ¹⁸FDG in the various astrocytoma types (I-IV). The question to be considered here is whether or not the glucose metabolic rate in the various glioma types is different depending upon the differentiation of the neoplasm. Also the assessment of borderline cases, i.e., patients in whom the clinical suspicion of brain tumor is not convincingly validated by neuroradiological findings, could be facilitated using this technique.

B) Regional cerebral glucose consumption using ¹⁸FDG in epilepsy. Of particular interest are studies of epileptic patients presenting with EEG and ECoG faci and negative neuroradiological tests (CT, RNS, CAn, PEG). Also, patients with deeply located lesions and normal or nonlocalizing EEG findings represent an important group to evaluate by this method.

C) Positron emission tomography to determine regional cerebral metabolism at rest and during cognitive or pharmacological stimulation in patients with Alzheimer's disease, Huntington's disease and those at risk for Huntington's disease.

D) Regional cerebral glucose consumption using ¹⁸FDG in the various stages of stroke, as well as TIA. There are reasons to believe that ¹⁸FDG PET should be more sensitive than conventional CT, radionuclide brain scans (RNS) and cerebral angiography (CAn) in the evaluation of the stroke patient. There is already some evidence of mismatches (non-coupling) between flow and glucose utilization. In cerebrovascular lesions, a differentiation between ischemic (reversible) and infarcted (irreversible) lesions can perhaps be made.

E) Regional cerebral glucose consumption using ¹⁸FDG in edematous regions of the brain. The critical aspect of this study will be an analysis of the func-

tionality of the edematous cerebral tissue. The areas of edema (surrounding tumors, inflammatory processes, MS plaques, bleedings or other edema-generating foci) will be recognized by conventional CT. Comparative analysis of the glucose metabolic rate in the edema areas vs. control regions could be carried out.

F) Regional cerebral glucose consumption using ^{18}F FDG in the brain of patients affected by Parkinson's or Huntington's diseases to establish the presence and extent of disturbed glucose metabolism in the various overt stages of these two pathological conditions and, for Huntington's disease, in patients at risk.

G) Regional cerebral glucose consumption using ^{18}F FDG in degenerative diseases of the brain, particularly in leukoencephalopathies. At present we are involved in a comprehensive study of a large variety of leukoencephalopathic processes. It would be most interesting to assess the glucose metabolic rate of the leukodystrophic regions. Also, in some cases, the differentiation between periventricular leukomalacia and transependymal CSF migration (hydrocephalus) may be difficult and of critical diagnostic importance. Perhaps ^{18}F FDG PET may prove useful here.

H) PET would permit a detailed analysis of flow, distribution and destiny of intrathecally injected CSF tracers. In conventional cisternography this detailed analysis is difficult for the deep regions which, on the other hand, are well suited for PET assessment. The anticipated improvement of CSF dynamics appraisal by PET has far-reaching implications for the study of such common pathological entities as hydrocephalus, dementia and other psychiatric disorders. As a tracer for CSF PET, ^{68}Ga DTPA appears to be an optimal choice (see FDA-approved-for-cisternography InDTPA).

I) We have some expectation that with high-resolution Neuro-PET we may possibly be able to evaluate the flow and metabolic rate (glucose) of the cervical spinal cord.

J) ^{18}F -DOPA could be used for direct external measurement of storage, degradation and turnover of intracerebral dopamine. The implication of the possible usage of this or other (^{18}F haloperidol, ^{18}F serotonin) radiopharmaceuticals for the external evaluation of the regional catecholamine metabolism will be far reaching.

K) Very preliminary reports indicate that the protein metabolic rate could be explored using tagged amino acids (such as valine) very much in the same fashion as the tagged deoxyglucose is used for evaluation of the original cerebral glucose consumption rate.

Group 2

A) Measurement and validation of regional cerebral blood flow (rCBF) using one (or several) method(s) possible with cyclotron produced radionuclides. The compounds suitable for this purpose include C^{15}O_2 , $^{13}\text{NH}_3$, $^{13}\text{N}_2\text{O}$, H_2^{15}O , ^{11}C -iodoantipyrine. The cyclotron produced $^{77}\text{K}_2$ has also been used for rCBF

detection. Actually the validation of a PET method to study rCBF represents an indispensable prerequisite for many other physiologic studies. A satisfactory blood flow agent is still to be agreed upon: for instance, the distribution of $^{15}\text{NH}_3$ is believed by one team of investigators to be related and informative about rCBF, whereas members of another team dismiss $^{13}\text{NH}_3$ as an agent to study rCBF on the basis that this compound perfusion is affected by metabolic variations and pH changes.

B) Measurement of cerebral blood volume (CBV) using ^{11}CO or C^{15}O .

C) Use and validation of ^{11}C -deoxyglucose (^{11}C -2DG) with its advantages over ^{18}F -fluorodeoxyglucose (^{18}F -2F2DG) for determination of local cerebral glucose utilization in man in normal and pathological conditions.

D) Measurement of regional cerebral O_2 metabolic rate using $^{15}\text{O}_2$.

E) Measurement and validation of regional oxygen extraction rate using the $^{15}\text{O}_2 + \text{C}^{15}\text{O}_2$ method (ratio of the two compounds equilibrium values).

F) Measurement and validation of method using ^{11}C labeled essential precursor aminoacids to study protein synthesis and turnover in the brain. From the nuclear medicine literature, promising aminoacids for this type of study are methionine, phenylalanine, tryptophane, leucine, and particularly valine.

G) Measurement and validation of functional localization of precursors of neurotransmitters, neuroreceptor agonists and antagonists, and distribution-turnover of neuroleptic drugs.

H) CSF. Studies on the CSF circulation based on radionuclide cisternography and, more recently, CT cisternography (with metrizamide) have reached an impasse. Discordant observations and opinions are ubiquitous in the pertinent literatures. The fact is that the CSF flow (third circulation) as well as the diffusion-transport of the various substances (proteins, aminoacids, metabolites, catecholamines) brain \leftrightarrow CSF are still scantily understood. The blood-CSF barrier and the related appealing CSF "sink" theory need to be elucidated. The implications of studies in this area for a better comprehension of the various types of dementias (and not only the dementia related to normal pressure hydrocephalus) are far-reaching. ^{11}C , ^{13}N , ^{15}O labeled physiological CSF components may be followed by PET as they move from and to the brain and its surrounding vascular structures. Proteins, aminoacids, metabolites such as homovanilic acid, 5-hydroxy-indole-acetic-acid, catecholamines as well as certain compounds which have a great capability of passing through the blood-brain-CSF barrier (e.g., aminonitriles which have already been ^{11}C labeled) represent some of the substances which may be used. They may be introduced intrathecally or systemically. Labeled drugs, already used or potentially usable for intrathecal therapy, are another group of compounds which would be worthwhile to follow in their passage through the CSF pathways.

Proposed Course of Project: The clinical studies with FDG-PECT have been initiated. Experience needs to be accumulated in the shortest possible period.

The collected observations have to be analyzed and interpreted. Construction of the new high-resolution PECT-scanner, the Neuro-PET, will continue.

Publications:

Brooks, R. A., Sank, V. J., Di Chiro, G., Friauf, W. S., Leighton, S.:
Design of a high-resolution positron emission tomograph: The Neuro-PET
J. Comput. Assist. Tomogr. 4:5-13, 1980.

Brooks, R.A., Sank, V. J., Di Chiro, G., Friauf, W. S., Leighton, S.:
Design considerations for high-resolution positron emission tomography.
IEEE Trans. Nucl. Sci. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01526-13 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Mechanisms of Epilepsy		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; padding: 10px 0;"> PI: Choh-Luh Li Medical Officer SN NINCDS </div>		
COOPERATING UNITS (if any) NONE		
LAB/BRANCH Surgical Neurology		
SECTION Neurophysiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.0	PROFESSIONAL: 0.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; padding: 5px 0;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; padding: 5px 0;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been temporarily suspended because of lack of assistance or supportive personnel.		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02010-08 SN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Neurophysiological Mechanisms of Pain		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> P.I.: Choh-Luh Li Other: 1) Rose Mary Borke Assistant Professor 2) Choh-hao Li Director and Professor </div> <div style="width: 30%;"> Medical Officer USUHS </div> <div style="width: 30%;"> SN NINCDS </div> </div>		
COOPERATING UNITS (if any) Department of Anatomy, USUHS Hormone Research Laboratory, University of California, San Francisco, CA		
LAB/BRANCH Surgical Neurology		
SECTION Neurophysiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">0.6</div>	OTHER: <div style="text-align: center;">0.5</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) Extracellular and intracellular responses of the ganglion nodosum to stimulation of the vagus nerve were recorded. Based on the conduction velocity of these extra and intracellular responses, the ganglionic cells appeared to respond mainly to the impulses initiated from the C-fibers; and, occasionally also from the A-delta fibers of the vagus. Investigation of the electrical properties of the ganglionic cells (e.g., input resistance, capacitance, time constance, change in membrane potentials and discharge activities) in response to electric stimulation of the vagus and superficial peroneal nerves and to application of chemical agents is in process. At present, no conclusive statements can be made.		

Project Description:

I. Objective:

- (a) To investigate the neurophysiological mechanisms of pain sensation.
- (b) To study the effect of conditioning somatosensory inputs upon the response of neurons which are activated by pain-fiber stimulation.
- (c) To study the effect of change in neurotransmitters in relation to the response of neurons activated by pain-fibers stimulation.

II. Methods Employed:

- (a) Adult cats under light general anesthesia were used.
- (b) The vagus nerve (right or left) and the ganglion nodosum were exposed.
- (c) The vagus nerve was placed in a specially constructed stimulating-recording chamber while the ipsilateral superficial peroneal nerve was similarly exposed, stimulated, and recorded.
- (d) The ganglion nodosum was penetrated by either a single or multibarrel micropipette electrode. The responses and changes in membrane properties of the ganglionic cells were recorded before and after nerve stimulation and application of pharmacological agents through iontophoretic injections.
- (e) Similar investigations are to be carried out in the nerve cells of the nucleus tractus solitarius nucleus ventralis posterior medialis of the thalamus and the sensory cortex.
- (f) The pain fibers and the neurons with dendrites and axons involved will be studied by horseradish peroxidase and electron microscope.

III. Major Findings:

There are no conclusive statements that can be made at this time.

IV. Significance to Biomedical Research and the Program of the Institute:

The present study will eventually provide a better understanding of the mechanisms of pain.

V. Proposed Course of the Project:

To continue the present study and to reorganize the laboratory by requesting a full-time and a dedicated technician to facilitate our research programs.

VI Publications:

None

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Central Nervous System Studies

National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980

Laboratory of Central Nervous System Studies
National Institute of Neurological and Communicative Disorders and Stroke

The Laboratory of Central Nervous System Studies comprises two major projects: (1) Neurobiology of Population Isolates--the Study of Child Growth and Development, Behavior and Learning, the Disease Patterns in Primitive Cultures; and (2) Chronic Central Nervous System Disease Studies--Slow, Latent and Temperate Virus Infections. Both projects are an outgrowth of the Study of Child Growth and Disease Patterns in Primitive Cultures. It was this parent project that gave rise to the discovery of kuru, a heredofamilial subacute progressive degenerative disease of the central nervous system of the Fore people in the Eastern Highlands of Papua New Guinea, and led to the demonstration that kuru is caused by a serially transmissible virus which possesses unconventional biological and biochemical properties. The successful transmission of kuru and the isolation of its virus provided the necessary techniques for the subsequent discovery of a viral etiology for some forms of presenile and senile dementias of man, particularly the Creutzfeldt-Jakob type, and it was this study that has led to the discovery that the agents causing these diseases form a group of transmissible virus-like agents new to the field of microbiology.

Following the convening of a series of international workshops on the "Subacute Spongiform Virus Encephalopathies and the Structure of the Unconventional Viruses Which Cause Them" held in the latter part of the last fiscal year, the staff of LCNSS participated in an international symposium on "Slow Virus" sponsored by NIAID and held at the Rocky Mountain Laboratory, Hamilton, Montana. Eleven papers were presented and have been published in bound volumes (Academic Press) resulting from the meeting; they covered the origin of slow infections in humans, the worldwide epidemiology of these diseases, the pathogenesis and molecular biology of the viruses, the biological, physical and chemical properties of the viruses including the evidence for strain variations and their unusual resistance to gamma and ultraviolet radiation.

Consistent with our earlier predictions, the most challenging outcome of the discovery that some chronic progressive noninflammatory CNS diseases (sporadic, as most cases of Creutzfeldt-Jakob disease (CJD); epidemic, as kuru; or familial, as familial CJD and kuru) are "slow infections" caused by viruses with incubation periods measured in years or decades, has been the realization that the etiologic agents of these infections are a new kind of micro-organism. Their unusual resistance to ultraviolet and ionizing radiation, to formaldehyde-propiolactone, and to heat, place them in a group unique among viruses. Their ability to produce fatal CNS disease without eliciting inflammatory responses, the failure of the course of disease or incubation period to be influenced by immunosuppression, and failure to demonstrate any antigenicity in high titer infective virus preparations, or to find any evidence of humoral or or delayed hypersensitivity reactions in the diseases, as well as an absence of response to interferon, stimulation of interferon, stimulation of interferon, or interference with interferon production, and absence of interference with known viruses, form the series of atypical biological properties which likewise

differentiate these agents from any other group of microorganisms. On the other hand, by demonstrating classical virus properties, such as adaptation to new hosts, broadening of host range and reduction of incubation period, dependence on the genetic breed of the host, the presence of strains of differing virulence in wild stock viruses selected by limiting detection, and the interference of fast-growing by slow-growing strains of scrapie, are all indicative of a complex host-virus genetic interaction characteristic of more classical viruses. Attempts to delineate the chemical nature of the replicating agents, especially to determine whether they are replicated from introduced genetic information or by the induction, derepression or activation of pre-existing genetic information in the host, are the major thrusts of current investigation.

The elucidation of the structure and molecular configuration of the infectious agent of scrapie, CJD, and kuru remains the first goal of this laboratory. For two decades this frustrating problem has been a challenge to molecular biologists, biochemists, and virologists.

In the past year we have made advances in our attempts to characterize the scrapie agent: (1) Cesium chloride fractionation of the infectivity. The general trend of the infectivity distribution in the first sedimentation to equilibrium from homogeneity of the mouse scrapie agent from a mouse brain homogenate has been determined. The infectivity is banding in a broad peak centered around density 1.24. The broadness of the peak indicates a considerable heterogeneity in density. Due to the steepness of the gradient we have achieved a marked separation from other components assayed, i.e. RNA, DNA, protein and lipid. Of greatest interest is the 500 x purification with respect to total brain DNA.

Individual or combined fractions from these gradients have been assayed analytically for scrapie specific DNA, RNA and proteins by gel electrophoresis but as yet without detecting a new species of macromolecule. The highly complex protein patterns are virtually identical in normal and affected brain except for several protein deficiencies in the affected animal.

The preliminary infectivity data also indicate that the cesium chloride gradient has concentrated the infectivity relative to a sample stored in cesium chloride and not banded. Study of the behavior of scrapie infectivity with exposure to high energies of sonication with rise in infectivity titer and fall even on frozen storage thereafter, indicate "sticky" clumping of the infectious units. Theoretical reinterpretation of much of the scrapie inactivation data in the light of the newly proved association or aggregation of infectious units indicates that even the aberrant behavior to UV and ionizing radiation may still be consistent with a larger virus than we previously suspected.

(2) Adaptation and development of the hamster 263-K strain of scrapie. Scrapie-infected hamster (strain 263-K) is a more suitable source of virus for purification studies. It is associated with a short incubation period and high initial titer of infectivity. The disease can be detected behaviorally only 55 days after a high titer passage, compared with a minimum of 180 days in the mouse system. Several titrations of hamster 263-K brain homogenates have consistently shown initial brain titers of $2-5 \times 10^{10}$ infectious units/gram of brain, over 100 times the titers obtained from mice. In a detailed analysis for biochemical studies and titration purposes, the hamster system is at least two times and, for some purposes, over 500 times more efficient with respect to

titration time and required animal space than is the mouse system. In terms of macromolecular distributions the hamster brain has fractionated much the same as the mouse brain. There is also a pronounced dependency of incubation time in the hamster on the dose of the agent, and this feature of the disease can be exploited to give an early indication of the distribution of the agent in fractionations, if not a quantitative assessment of infectivity.

Additional work focused on the possibility of obtaining infectious nucleic acids from extracted brain tissue. In order to enhance the potential infectivity of any naked nucleic acid recovered by our procedure we coupled the infectious assay with a transfection procedure which we had shown to be effective for herpes simplex virus. The experimental approach was to fractionate infected mouse brain homogenate following a heat inactivation step (80°C for 30 minutes) designed to inactivate any enzymes that might interfere with the recovery of infectious material.

Following heat inactivation the homogenate was digested with Protease K, then extracted with phenol in the presence of 1% sodium dodecyl sulphate (SDS). The resulting three fractions (aqueous, phenol and heavy interphase) were further extracted under conditions designed to preserve the molecular nature of the material finding its way to that fraction. The aqueous phase was further extracted with organic solvents and alcohol precipitated. The phenol phase was buffer extracted to recover any material and the interphase was buffer extracted to remove the phenol. The resulting fractions were assayed for infectivity in NIH Swiss Webster mice. The results of this experiment clearly indicated that there was no infectivity associated with the nucleic acid fraction. The conditions used in these experiments yielded infectious HSV-1 DNA from infected cells but provided no scrapie infectivity. The heat and Protease K treatment had no effect on the infectious titer, however the subsequent steps destroyed virtually all of the infectivity. The only possible infectivity should have been in the highest concentrations of the buffer extracted interphase from the phenol extraction; the presence of infectivity in this fraction has not been confirmed by pathology. These results suggested to us that the viroid model, at least in its simplest forms, is not valid for the unconventional agents. Further studies on the scrapie system have focused on our impression that an essential, very hydrophobic protein is intimately associated with the scrapie agent and that new procedures are necessary for its isolation.

More recent studies reported in the literature indicate that at least a small percentage of the scrapie population has a DNA component of low molecular weight that is DNAase sensitive which is eluted at 0.48M phosphate buffer from hydroxyapatite columns. This would suggest that the DNA molecule could be double stranded. During this year we tried to detect double-stranded scrapie-specific DNA by molecular hybridization experiments since analysis of the kinetics of DNA reassociation has proven to be a very sensitive means of detecting the presence of specific DNA sequences in mammalian genome. As a probe we used the DNA extracted from concentrated enriched scrapie labeled with 125 I and annealed to total DNA extracted from infected and uninfected brains of the same and different species. No difference was observed between the extent of reassociation of the probe with DNA of scrapie or normal animals. Our levels of detection indicate that if the scrapie agent were a double-stranded DNA molecule its presence in infected brain tissue is below the level of 50 molecules of DNA per infective unit. We have sought also to repeat the work of others claiming to have isolated a scrapie-specific DNA. However,

our attempts to reproduce this much discussed procedure are dissappointing with less than a 1% recovery of infectivity in the high speed supernatant as opposed to the 10-90% indicated by Marsh and Malone. When this high speed cell-free virus was placed on a 2.5% polyarylamide-0.5% agar rose gel (9.5x0.6cm tube) at 6 mA of voltage for 2 hours, all of the infectious virus entered the gel and was recovered (4.8×10^6). Enzyme treatment of these infectious units was not interpretable due to the total inactivation of the virus at 37°C after 3 hours. These studies are being continued.

In yet another approach we have been comparing neurotransmitter concentrations in brains of scrapie-affected and normal mice and hamsters in the hope of identifying a particular neuronal system as the target for the infection in the brain. Comparing late scrapie mice with same age controls we have observed normal levels of catecholamines and most amino acids, but a two-fold increase in GABA levels and a nearly 100-fold decrease in 5-hydroxytryptamine (5-HT) levels. This finding prompted us to look at 5-HT levels in the blood. In the case of late hamster scrapie we observe a somewhat variable but significant decrease in blood serotonin of almost two-fold. At present these findings are being vigorously pursued: (1) to discover the time course of these changes and correlate them with behavioral changes and histopathology; (2) to narrow down by behavioral neuropharmacology, and brain microassay of neurotransmitters and enzymes the specific lesion(s) involved; (3) to identify other non-CNS indicators of these changes which may be of clinical use; and (4) to test the efficacy of 5-HT analogs as a therapy.

Our studies on the therapeutic benefits of the serotonin agonist, quipazine maleate, and the serotonin precursor, L-5-hydroxytryptaphan methyl ester, on scrapie infectious hamsters have shown that both drugs effect small but statistically significant improvements on ataxia and action jerks within a rather narrow dose range. At higher doses we observed a dramatic hypersensitivity in the scrapie infected animals to the toxic effects of both drugs. This hypersensitivity syndrome is an intensively studied phenomenon in the rat and has been shown to originate in that system from neuropharmacological destruction of serotonergic nerve terminals. The hypersensitivity that we have observed in the hamster is even more than that which can be induced by neurotoxic agents in the rat. Thus we may support that the scrapie infection in the hamster results in the destruction or degeneration of the axon terminals of the serotonergic nerves. This is the first example of a serotonin hypersensitivity arising as the consequence of a natural disease state.

In our studies of the biochemical levels of serotonin in the brains and blood of scrapie infected hamsters and mice we have observed the following: (1) a highly significant 2.5-fold decrease in the blood serotonin levels in scrapie infected hamsters but no similar change in mice; (2) a highly significant 20% reduction in mouse brain serotonin levels but no similar change in hamsters. This change in mouse brain concentrations is seen only in the late clinical stage of disease; and, (3) a much larger 10-fold decrease in mouse brain serotonin levels after frozen storage for a prolonged period. Our observation of a 2.5-fold decrease in blood serotonin levels in scrapie infected hamsters is the first major change in blood chemistry noted in the subacute spongiform virus encephalopathies.

In a continuing effort to both characterize the agent and find ways to inactivate and/or stabilize it we have performed the following inactivation

experiments: (1) sensitivity of scrapie to shear forces; (2) sensitivity of scrapie to osmotic shock; (3) sensitivity of scrapie to exhaustive protease treatment; and (4) sensitivity of scrapie to chlorine dioxide.

Results of these studies show: (1) overall scrapie infectivity in brain homogenates can be increased at least 17-fold by exhaustive sonication immediately prior to titration. This quantifies to some extent the level of aggregation of scrapie virus in the usual preparations. We have extended these studies to determine whether or not the high intensities of sonic radiation used in these experiments are inactivating infectivity as well as dissociating aggregates as well as investigating the kinetics of reaggregation. (2) Much of the infectivity loss often associated with exposure to high ionic strength buffers is apparently due to enhanced aggregation under these conditions. (3) If scrapie is inactivated at all by powerful proteases this occurs at a much slower rate than for brain homogenate proteins in general. (4) A kinetic analysis of the inactivation of scrapie infectivity by sodium hypochlorite and chlorine dioxide, show both chemicals to be equally effective inactivating 99.9% of the population in the first few minutes of exposure.

A critical analysis of ionizing radiation data and electrophoresis of scrapie has been undertaken during this past year. The conventional wisdom is that the infectious agents of the subacute spongiform virus encephalopathies (SSVE) are very small, probably even subviral in size. A favorite hypothesis is that they may represent examples of animal viroids. This expectation is based upon the well established resistance of the SSVE to inactivation by ionizing radiation and, more recently, the observation that scrapie infectivity will comigrate with a viroid marker in some electrophoretic gel system. Dr. Rohwer in our laboratory has now offered intriguing alternative interpretations for both of these findings. He has shown that if the SSVE are highly aggregated, as his sonication data indicate (see above), then the traditional first order analysis of the ionizing radiation data is inappropriate. If aggregation is taken into account in the analysis of the inactivation kinetics, the actual size of the scrapie agent must be much larger than that deduced previously from a first order inactivation constant and, in fact, is consistent with the molecular weight of ordinary viruses. Dr. Rohwer has also shown that, in the electrophoretic systems used to characterize the mobility of the scrapie agent, viruses fractionate on the basis of their charges whereas nucleic acids fractionate on the basis of their molecular weights. In these same systems simple bacteriophages comigrate with much smaller nucleic markers and in fact the two species cannot be used to calibrate one another and separations such as these cannot distinguish viruses and viroids.

During the period covered by this report major efforts have been made to study the interaction of scrapie with the immune system of infected animals. These studies have been done in three parts. First, the search for a new antigenic component on the surface of spleen cells at various times following infection. Second, a systematic examination of the interaction of scrapie with a C3H/HeJ mouse line reported to be unique. Thirdly, the identification and culture of the infectious cell population in the mouse spleen.

The search for a new antigenic component of the surface of spleen cells was based on the possibility that a new cell surface component would not be detected by the humoral immune response but would be detected by the cellular immune system. To examine this possibility, mixed lymphocyte cultures were utilized

using two inbred strains of mice, Balb/C and C57BL/6. Two large groups of animals were studied with cultures at weekly intervals over the early and late stages of infection. In every case controls inoculated with normal mouse brain were included on a 1:1 ratio. Data on the early times post infection included spleen weights to check for the enlargement reported by others. Throughout this study the results were uniformly negative with respect to both the splenomegaly and to the presence of any new cell surface component. Several cultural combinations were included to examine the scrapie-infected cells as both target cells and responder cells. It seems clear from this work that: (1) there is no new cell surface component on scrapie-infected spleen cells that can be detected in mixed lymphocyte culture; (2) scrapie-infected spleen cells retain the capacity to respond to the mitogens Con A and LPS as well as respond to a heterologous H-2 determinant in mixed lymphocyte culture. These responses are identical in magnitude to those animals inoculated with normal mouse brain; (3) there is no detectable splenomegaly in scrapie infected mice within the first three months of infection and the data suggest that there is no splenomegaly throughout most infections.

Extensive studies with the C3H/HeJ strain of mouse have not confirmed the published report of other investigators that this strain of mouse, when infected with scrapie, loses its ability to mount a mitogenic response to the endotoxic protein component of *E. coli* LPS. This animal is genetically unable to respond to the Lipid A moiety. These studies were carried out at weekly intervals from weeks 2 through 7, since previous reports indicated the peak depression to occur at week 4. It has been reported that a marked spleen enlargement occurred, a finding also not confirmed in this work. There are only two possible explanations for the lack of agreement--one is a difference between the Chandler and C506 strains of scrapie, or that other investigators had a contaminating virus in their inocula. The plan for the future is to attempt to determine which of these is the explanation and to attempt to clarify completely if there is or is not a measurable change in the immune response of C3H/HeJ mice with scrapie.

The results of the spleen cell sub-population studies have been completed. It is clear that strain C506 gives extremely low spleen titers and that only a very small number (less than 1 in 10^5) spleen cells are infectious, whatever sub-population they are in. Extensive studies on splenic macrophages have been disappointing from the point of view of continued infectivity.

We have also explored the ability of scrapie to grow in vitro in well-established, 'T', 'B' and macrophage cell lines of murine origin. Two questions are being investigated: (1) does the cell have a receptor for scrapie on their cell surface?; and (2) if they do not have the receptor (assuming that scrapie agent is the free nucleic acid bound to lipid membranes), other methods have to be used to get the agent in the cell so that it could replicate. Inactivated Sendai virus and lysolecithin were used as membrane-fusing agents; DEAE-Dextran, which alters the permeability of the membrane and is used for assay infectivity of other viral nucleic acids in cell culture, was also used. Cell culture harvests from these experiments have been titrated in mice for infectivity and the results from these experiments will help us answer the two questions. Since most of the murine cell lines used in the study have endogenous C-type viruses, it will also be interesting to see if these viruses act as helper viruses for the growth of scrapie. Attempts to grow scrapie in mosquito cells: *Aedes Albopictus* mosquito--cell lines have been used to grow several groups of

arboviruses. In such cells these viruses grow at 22°C without producing cytopathic effect, and infected cells become chronically infected by the virus. Virus is released from these chronically infected cells into the medium. We have infected these cells with the scrapie agent, and cell lysates at different passage levels have been inoculated into mice for the assay of infectivity. Results were discouraging since unlike some members of the togaviruses, scrapie infectivity was not recovered from inoculated insect cell lines. An SV-40 transformed cell line that contained scrapie virus at the 12th passage level was serially passaged to higher levels; none of 50 pooled and cloned cultures was infectious for mice at the 30th passage level or higher. The scrapie-infected SMB line of Clarke and Haig was imported from England; five lots of this line have been prepared and aliquots stored; mutants of the cells are being prepared. A line of cells was derived from the brain of a hamster infected with the 263-K strain of scrapie; this line is also under study.

Since conventional immunological techniques have thus far failed to elicit an antigen-antibody reaction in either kuru, Creutzfeldt-Jakob disease or scrapie, we have been attempting to produce specific antibody to scrapie by the hybridoma technique of Kohler and Milstein since it has been shown that cells from a mouse myeloma could be fused with splenic cells from mice stimulated with an antigen, and such fused cell clones produce specific antibody which is monoclonal for individual antigenic determinants. Such a technique facilitates antigenic analysis of complex antigens. In our studies spleen cells from mice immunized with scrapie infected mouse or hamster brain scrapie specific antibody has not yet been obtained; however, 30 monoclonal antibodies were derived which are reactive to antigens in hamster or mouse nervous system tissues. Of the 30 clones analyzed, specificity included clones reacting with grey matter of mouse and hamster brain, one clone reacting with axons in animal brain, several clones reacting with cytoskeletal proteins (intermediate and micro-filaments) and 19 clones which produced antibody reactions with both neural and non-neural tissue components.

We also measured the general immunocompetence of splenic lymphocytes in an attempt to detect alterations of the immune system of scrapie affected animals. In general splenic activation by Concanavalin A, phytohemagglutinin and lipopolysaccharide of control and scrapie inoculated mice were compared. Mitogen-induced responses of splenocytes from infected and control cultures were not significantly different. The PHA response of scrapie-infected mouse spleen cells was slightly depressed over a period of 29 to 56 days post-inoculation. Additional efforts to induce scrapie specific antibody are underway and indeed the use of several different preparations of high-titering scrapie infected hamster brain that has been subjected to (a) chemical tissue membrane modifiers, (b) purified by density gradient banding, and (c) tied up with haptens. Such mitogens are being assayed in animals rendered immunotolerant to uninfected hamster brain.

As a control for the scrapie studies, somatic cell hybridization to produce monoclonal antibody against a major glycoprotein (P₀ 30,000 MW) associated with human peripheral nervous system myelin was carried out. Thus far we have produced two clones both of which react with peripheral nerve myelin; only one produces antibody specifically reactive with the P₀ low molecular weight glycoprotein.

Since the demonstration of cell-fusing activity in the majority of brain extracts of scrapie mice and CJD patients (see ANNUAL REPORT: October 1, 1977 through September 30, 1978) additional studies have been carried out using two different techniques. One involved the formation of multinucleated cells and the other the formation of somatic hybrid cells. Heterokaryons were measured at 18 hours and hybrid cells after an average of 25 days. The studies employed three scrapie cases, 32 cases of transmitted CJD, two cases of untransmitted CJD, 26 cases of other neurological diseases, three transmitted cases of other than CJD and 17 patients without neurological disease. The results show a significantly higher proportion of CJD brains (61%) was positive than other neurological diseases (31.4%) or the control group (6%). Thus our earlier observations have been clearly confirmed and although the assay does not separate CJD from other neurological diseases to warrant its use as a specific diagnostic test we hope that such discrimination can be improved to the extent that the detection of cell-fusing activity might be possible utilizing serum, urine and CSF from patients and their family members as a biological marker of this disease. We shall continue to study the phenomena of cell fusing activity in an effort to elucidate the mechanism in CJD and other neurologic diseases as well as the application of this technique as a rapid means of more quickly measuring infectivity in experimentally derived fractions from purification procedures employed for scrapie and CJD.

Recently study of the appearance of this cell fusing activity in brain of hamsters infected with scrapie has shown peak fusing activity attained early in incubation (4 weeks) instead of during clinical disease (8 to 9 weeks). This may indicate the desirability of studying hamster brain early in the incubation period for possible biochemical markers of scrapie virus or scrapie activity.

Resistance to high concentration of formaldehyde, to heat up to 85°C, and to ultraviolet radiation at 254 nm, and an ultraviolet sensitivity at 237 nm greater than at 254 nm have been found for kuru and CJD viruses as for scrapie. These very unusual physical properties greatly emphasize our current contention that the viruses of the human diseases are closely related to the scrapie virus. Similarly, the two human agents have been shown to have the same enormous resistance to ionizing radiation (gamma rays from Cobalt C060 as is found for scrapie virus. The most direct inference from this enormous resistance is an effective size of under 100,000 daltons molecular weight. Although many possible explanations, including atypical fine structure for a nucleotide configuration and unusually efficient nucleic acid repair mechanisms have been suggested to account for such anomalous properties, the simplest explanations namely, that in fact the agents are of such small size, may be true; or, the new data of extensive "sticky" clumping or aggregation of infectious units may account for much of the anomalous behavior.

The discovery that the worldwide-distributed Creutzfeldt-Jakob disease is caused by a serially transmissible, self-replicating agent that passes through bacteria-, protozoan- and fungus-retaining membrane filters, the demonstration that the virus is widely distributed in tissues and fluids outside the CNS of affected patients and possesses the physiochemical properties as described above, has also resulted in a growing concern among medical and paramedical nursing and laboratory personnel, particularly neurologists, neurosurgeons, pathologists, and anesthesiologists, about the potential hazards involved in caring for patients with presenile dementias and handling their tissues. Concern comes largely from recent reports documenting transmission of

Creutzfeldt-Jakob disease by corneal transplant, the accidental inoculation of two patients in neurosurgery with CJD-contaminated electrodes used in stereotactic electroencephalographic recording and stimulation, the suspicion that a neurosurgeon and two general practitioners may have contracted CJD from patients and the characteristic greatly over-represented among patients with CJD of a history of brain or eye surgery in the previous two years before onset of clinical disease. These concerns have further been heightened by the recent transmission of CJD to a chimpanzee by implantation of the same silver electrodes that had caused disease in the two human patients after more than two years storage in formaldehyde vapors used for sterilization. In response to these concerns we have published precautions for conducting biopsies and autopsies and have more recently, presented a summary on the current knowledge of the pathogenicity and communicability of CJD and related subacute spongiform virus encephalopathies of man and animals which are caused by similar unconventional viruses. We have also made recommendations on the rational precautions that should be taken in caring for these patients and in handling their tissues.

During the last year, inactivation studies were made with disinfectants using mouse scrapie agent. Mouse scrapie, kuru, and CJD agents seem to have similar properties. Disinfectants used were clorox, organic iodine (Wescodyne), potassium permanganate, hydrogen peroxide, and Zepharin. Since ethylene oxide gas is commonly used in hospitals, ethylene oxide was also used. The data showed that a 1:250 dilution of KMNO_4 and a 5% solution of clorox are the most effective disinfectants, followed by Wescodyne and ethylene oxide which reduced infectivity by 99 percent. Under the experimental conditions used in the study hydrogen peroxide did not affect the titer of the scrapie agent at concentrations used in the hospital environment. Residual toxicity of Zepharin for mice was too high to draw any conclusions. Further studies are in progress on the CJD agent, with ethylene oxide autoclaving used for sterilization in the hospital setting. Finally, chlorine dioxide has been examined in parallel with potassium permanganate for inactivation activity against a guinea pig-adapted strain of CJD virus; and chlorine dioxide, sodium hypochlorite, potassium permanganate, hydrogen peroxide, and lysol® have been tested for activity against a hamster-adapted strain of scrapie. Time-dose experiments are on titration at this time, and should be completed within the year. Depending upon the results further recommendations will be made to the medical community.

In an effort to determine the method of spread of CJD virus in man, we have recently completed a comprehensive worldwide epidemiologic survey of CJD. It is shown that in the United States the average annual mortality is at least 0.26 deaths per million population. Temporal-spatial clustering of cases was not found in the United States, but reports from other countries indicate that this occurs. Fifteen percent of the cases were of the familial type, suggesting a genetic susceptibility to infection. In this survey, some evidence was found that previous surgery or pre-existing neurologic disease may be associated with an increased risk of developing CJD.

A systematic investigation of all cases of CJD dying in France during the decade 1968-1977 was completed during this year in collaboration with Dr. Francoise Cathala and members of the French Neurological Society, with a view towards clinical definition of a large and unselected case series, and to obtain some clue as to the natural mode of disease transmission. One hundred and seventy cases were discovered, of which 124, confirmed by autopsy or biopsy,

were the subject of multifactor statistical analysis. The disease forms a clinical spectrum from nearly acute encephalitic type illness with a few weeks' rapid progression and death, to lingering illness of years' duration, impossible to diagnose in the absence of neuropathological verification. Types of clinical onsets, range of symptoms during the course of illness, and symptom combinations with the highest frequencies were analyzed in detail. In addition, epidemiological data on all 170 cases were examined for the possibility of iatrogenic or case-contact types of human-to-human transmission. Apart from the approximately 10% of familial cases, no contact could be established between any two patients in France during a 10-year period, no iatrogenic transmission was discovered, no case occurred in any member of the medical profession, and those cases in paramedical professions did not occur at a higher rate than in the general population. Close examination of familial cases established that even in such families, personal contact between two subsequently affected members does not always occur, suggesting ever more strongly the participation of predominantly genetic factors in the familial type of CJD. Our epidemiological studies have already indicated that an annual incidence of nearly one case per million can be expected when newly occurring cases are actively searched out. The frequency of the disease continued to be highest in the densely populated center of Paris, raising further speculation about human-to-human modes of natural transmission. On the other hand, study of exceptionally isolated cases, which could simplify examination of the number of possible routes of acquiring the disease, still has not yielded any clues to this problem. A full-scale study of any possible association of CJD and scrapie in sheep is also under way.

A detailed analysis of the clinical features of the first 100 transmissible cases of CJD has been performed, and the results compared to the clinical features of a similar number of cases of Alzheimer's disease. There is a considerable overlap in the clinical spectrum of both diseases, and a group of patients with Alzheimer's disease with myoclonus has been delineated for further clinical and pathological evaluation. In addition, the clinical syndrome of "amyotrophic" CJD and a group of cases of "untransmissible" CJD are being studied.

Other clinical features of CJD which may be related to different strains of the virus are being examined. A manuscript is in preparation describing a small number of cases of CJD with the clinical features of progressive supranuclear palsy. The differences between the acute and chronic forms of CJD have already led to the discovery of a virus strain from a Japanese case that takes readily in non-primates and causes both gray and white matter spongiform lesions. The possibility that the virus also causes previously unrecognized childhood encephalopathies is also being investigated.

In a continuing investigation on the possible modes of natural transmission of the CJD virus, we are intensively evaluating the familial occurrence of the disease. To date, we have identified 37 families with a total of 155 affected members. CJD occurs in a pattern suggesting autosomal dominant transmission. Compared with the sporadic form of CJD, in familial CJD the age at death is slightly earlier and there is a female preponderance. The clinical and pathological features are otherwise indistinguishable. No maternal effect was found. There was some evidence for anticipation. An analysis of temporal and spatial separations between affected family members suggests that if contact transmission were occurring, incubation periods up to four decades might be expected. However, the available data do not yet allow us to distinguish

between a genetic susceptibility to infection or some form of vertical transmission. Studies are in progress determining genetic markers, such as the HLA type, of both sporadic and familial CJD, which might give us an indication of the genetic component of susceptibility to infection.

A major part of our experimental studies on CJD include the routine screening of the brains of all animals dying after inoculation with various chronic neurologic diseases, since it is now known that in the case of the squirrel monkey at least, approximately 15% of the animals die without showing clinical signs of neurological disease. The topography of the spongiform change has recently been analyzed in more than 200 squirrel monkey brains, where the results indicate that considerable variation in the severity and distribution of the lesions occur. The differences between CJD, kuru and scrapie are being examined in both primate and non-primate hosts. The unusual white matter change produced by a Japanese strain of CJD in mice is being examined.

A re-evaluation of the spongiform change in human kuru is being performed to see if the same general features as seen in human CJD also occur. The peculiar amyloid plaques that occur in 60% of kuru patients and approximately 10% of CJD patients is being investigated both structurally and at a biochemical level. The occurrence of these amyloid plaques in a virus-induced encephalopathy has great relevance to the etiology of the plaque of Alzheimer's disease.

With our demonstration of the transmissibility of scrapie disease from American sheep and English goats to several species of non-human primates, manifested by a disease in the experimental monkey that is indistinguishable from the transmissible virus dementia originating from man, we are confronted with the urgent question of the possible relationship between scrapie of sheep and the spongiform encephalopathies of man. The scrapie virus is capable of infecting all species of monkeys tested. However, the Compton (English goat) strain after passage through non-human primates no longer induces disease when inoculated back into sheep or goats. Of tremendous importance however has been the discovery that although these same strains of non-human primate-adapted scrapie virus did not induce clinical disease in mice during the more than two years they were observed, such mice did in fact have neuropathological lesions of spongiform encephalopathy in their brains and sub-inoculation of this material did induce disease in other mice. A similar observation has now been made on CJD in mice wherein transmission occurred on primary passage of human brain but on the first mouse to mouse passage animals remained asymptomatic for over 2-1/2 years yet when killed histopathological evidence of spongiform encephalopathy was observed in their brains. Thus, we have evidence that infected animals can remain asymptomatic and that in these animals the incubation period before onset of clinical disease may exceed the life span of the host.

The same exceptionally long incubation periods are evidenced in those few cases of kuru that have occurred in the Fore of Papua New Guinea during the past five or six years; new cases occur only in patients over 20 years of age.

The biological properties of scrapie appear to be altered after passage through the primate host-behavior, not unlike classical viruses; such altered biological properties may account for the failure of CJD and kuru viruses to induce disease in mice routinely. We have experienced difficulty in adapting the virus of CJD to mice and guinea pigs, but in recent experiments some passage

lines of CJD have caused spongiform encephalopathy in both guinea pigs and mice, and we have recently completed studies on the pathogenesis of the Japanese strain of the virus in Balb-C mice. The findings were strikingly similar to the pathogenesis of scrapie in the mouse with a few notable exceptions. Initially, characteristic spongiform degeneration of the brain was first noted pre-clinically at 9 weeks following inoculation. Clinical signs did not become apparent until 16 weeks with the geometric mean incubation period being 112 days. Infectivity assays of various tissues of inoculated mice resulted in recovery of virus from brain and spleen as early as one week after inoculation. Furthermore, the average incubation period of mice inoculated with spleen was markedly less than that of mice injected with brain material from the second through the sixteenth weeks of incubation indicating that the concentration of virus is higher in the spleen than in the brain during the asymptomatic period. Lesser amounts of virus were detected in thymus, lung, and kidney. In the kidney the virus appeared late in the pre-clinical period and the incubation period for recipient mice were prolonged. Virus was not detected in the liver in contrast to its presence in this tissue in human patients. Viruria was not demonstrable. However, we did confirm the presence of a viremia in CJD infected animals beginning during the sixth week after inoculation. Concentration of virus in the blood at the 14th and 18th weeks were estimated to be appreciable since the incubation periods in recipient mice ranged from 4 to 5 months. The clinical disease was confirmed histologically.

We have now proven the transmissibility of the spongiform viruses by the oral route through feeding of virus-infected whole tissues. Two of two squirrel monkeys fed CJD-infected chimpanzee tissues and two of two squirrel monkeys fed scrapie infected whole tissues developed clinical disease and had typical pathological lesions of the spongiform encephalopathy in their brains. One of two monkeys fed kuru-infected chimpanzee tissues developed spongiform encephalopathy. The asymptomatic incubation period in the one monkey exposed to kuru was 36 months; that in the two monkeys exposed to CJD virus was 23 and 27 months, respectively; and that in the two monkeys exposed to scrapie virus was 25 and 32 months, respectively. The one additional animal similarly exposed to kuru has remained asymptomatic during the 45 months it has been under observation.

The elucidation of the etiology and epidemiology of a rare, exotic disease restricted to a small population isolate--kuru in New Guinea--has now brought us to worldwide considerations that have importance for all of medicine and microbiology. For neurology, specifically, we have considerable new insights into the whole range of presenile dementias, and, in particular, to the larger problems of Alzheimer's disease, familial and senile dementias, and the processes of CNS aging. The implications of vertical transmission of slow virus infections, of conjugal transmission of these diseases, and of host genetic control of disease expression for all genetic diseases, and the relationship of these slow virus infection processes to those which may lead to neoplastic transformation are obvious.

The major problem among the degenerative diseases of multiple sclerosis, amyotrophic lateral sclerosis, and Parkinsonism remain unsolved, although there are tantalizing laboratory and epidemiological data pointing to the possible role of virus-like agents in these diseases. Perhaps the masked and defective slow infections with conventional viruses such as are seen in PML and SSPE may provide the best leads for studying these diseases.

Our scientific direction of the amyotrophic lateral sclerosis (ALS) studies at the Guam laboratory of NINDS for the study of the ALS-PD complex in high incidence among the Chamorro people, has resulted in some 12 publications which have already appeared, or are in press, and many promising ongoing studies. These are summarized below, but they indicate our conviction that the answer to the perplexing problem of motor neuron disease (ALS) and Parkinsonism-dementia (PD) are to be found in these ethnically and geographically limited foci.

Our study of the similarly intense focus of ALS and Parkinsonism and dementia among the isolated Jakai and Auyu people of Western New Guinea, discovered during our field studies (New England Journal of Medicine, 1963), and with two recently updated reports just published (Ciba Symposium, 1977; Symposium on ALS, February 2-3, Tokyo, 1978) is proceeding with further field work this year. Once again the intense localization of the focus in a small population and limited geographic area suggests strongly a restricted environmental variable (plant toxin or mineral substance or a deficiency) coupled, perhaps with genetic factors in the population. This year's work has proven that the disease is fully environmental and that ALS and PD are related as evidenced by (1) husband and wife with classical ALS; (2) husband with pure PD, wife with classical ALS, simultaneously; (3) next door neighbor to (2) above with classical PD; and (4) two women with classical ALS in 1974 in same village and a neighbor with PD. It appears that the "rule" is that people living or drinking exclusively from small springs and rivers originating in the "red-soil" lowland plain get ALS/PD. People of the same cultural and linguistic groups as these suffering from ALS and PD but living on tidal flats and on big rivers originating from the high mountains do not get ALS/PD. The water and soil analyses indicate extremely low calcium in garden soils and drinking water and the pattern of occurrences seem, as in endemic goiters to follow geological features of the environment rather than the patterns of ethnic and cultureal demographic distribution. With this in mind, we are covering these possibilities of mineral metabolism, imbalances and trace metal toxicity as well as those of an endogenous virus in an isolated population in our studies on Guam and West New Guinea.

We have increased our collaborative research with the Japanese investigators, who have been helping us on Guam by providing us each year with a young neurologist to assist in the clinical neurological surveillance and care of our patients there and in collaborative pathological, biochemical and pharmacological studies. During this reporting period, Dr. Takao Makifuchi, of the Brain Research Institute, Niigata City, Japan, took up residence on Guam as a Visiting Scientist; and now Dr. Kiyomitsu Oyanagi has arrived to replace him. Also, Dr. Richard Yanagihara was recruited for Guam, and after three months of intensive preparation and developing protocols here at NIH proceeded to Guam where he initiated a study of Ca, P, Mg and trace metal metabolisms including C47 calcium trace studies on ALS, PD and control subjects.

The Japanese are themselves concerned with their own foci of high incidence of ALS and PD on the Kii Peninsula of the main island of Japan. The series of meetings and conferences on ALS in Japan held in March 1978 resulted in the confirmation by Dr. Hirano of the pathological identity of the Kii Peninsula PD cases with those on Guam (both demonstrating neurofibrillary tangles), and the final agreement that the two disease foci represent the same disease complex. During his 1979 field studies in West New Guinea, the Chief, LCNSS, has obtained definitive evidence that classical Guamanian ALS, PD, and ALS/PD does occur in

the high incidence foci he discovered in West Irian and is very excited about resolving this problem. In addition, Dr. Gajdusek noted the occurrence in West New Guinea of a subacute progressive paralysis that looks like "slow-poliomyelitis" vitamin B deficiency. He has seen many cases this year and recognized it as the same disease he first saw in 1974-1976 field trips. The disease is not ALS; it can be "acute", it is often fatal, but remissions and recurrences do occur. A few cases have had beriberi-like edema with onset but most have not. That this very severe paralytic disease should occur within the ALS/PD focus is amazing. International collaboration and, most importantly, more original and innovative research concepts and more imaginative and cautious study of the various Western Pacific foci have continued and been expanded. Those studies which are underway in our collaborative project, and a bibliography of recent publications (1975-1980 in press) resulting from studies of these foci are included as an appendix to this annual report. The ongoing studies include:

- (1) Clinical variations in ALS-PD complex in Chamorros;
- (2) Human biology of ALS-PD complex and other chronic diseases in Chamorros of the Mariana Islands;
- (3) Chronic CNS disease and disability survey of Guamanian Chamorro migrants to the mainland United States;
- (4) Genetic studies of the Chamorro population, both normal and ALS-PD afflicted;
- (5) Detection of sedimentable reverse transcriptase activity in the brains of patients dying with ALS-PD;
- (6) Search for biochemical defects in ALS-PD brains by gel diffusion chromatography;
- (7) Search for nucleic acid repair mechanism defects in transformed leucocyte cell lines derived from ALS-PD patients;
- (8) Search for an ALS or PD specific antigen in brain tissues by clonal myeloma cell hybridization with spleen cells of ALS and PD from hyperimmunized animals and resultant monoclonal antibody production;
- (9) Trace aluminum and other heavy metal studies in brain, CSF, blood and other tissues of ALS-PD patients;
- (10) Evaluation of the precise nature of the cognitive and affective defects and the progression of dementia in the PD patient;
- (11) Evaluation of liver function and pathology;
- (12) Development of techniques for the unmasking of an infectious agent by in vitro techniques;
- (13) Assessment of the immunological competence of patients;
- (14) Attempts to transmit ALS-PD to non-human primates and non-primate hosts;
- (15) Major virus group seroepidemiology of the Mariana and Caroline Islands, Japan, and West New Guinea populations with relation to ALS-PD;
- (16) Pharmacologic studies of ALS-PD;
- (17) Elucidation of osteoporosis, osteoarthritis, and bone deformities in the Chamorros; and
- (18) Evaluation of the growth and development of normal Guamanian children and adolescents--a 30-year follow-up study.

The genetic studies, already well advanced, include blood group factors, red cell enzymes, serum proteins, HLA typing, and mixed leucocyte agglutinins, dermatoglyphics, anthropometry and other gene markers.

Since World War II, there has been an extensive migration from Guam of at least 15,000 Chamorros, primarily to the United States. This represents nearly one-third of the total Chamorro population of 47,000 residing on Guam. Amyotrophic lateral sclerosis has developed in 14 Chamorro migrants from Guam to the United States, Japan and Korea after periods of one to 36 years of absence from Guam. Nine of these cases have been previously reported. In another eight subjects ALS has developed within 1 to 14 years of their return to Guam after absences of many years from the islands. Parkinsonism dementia, a high incidence presenile dementia peculiar to Chamorro Guamanians, has developed in one subject 46 years after his departure from Guam. It appears that the onset of ALS in these patients after long absences from Guam will demonstrate the lower limit for the incubation period in each case if a toxic or infectious exposure occurring only on Guam is the cause of the disease.

Additionally, during the past two decades there has been an increasing number of cases of Guamanian ALS in long-term Filipino migrants to Guam. The average annual incidence rate of ALS in these migrants is approximately five-fold higher than the rate of ALS in the United States. Parkinsonism dementia-like disease has been clinically identified in five Filipino patients and one case with autopsy verified pathologically. Because of the high degree of genetic similarity between the Chamorro and Filipino peoples, which we have recently demonstrated, a detailed epidemiological survey for ALS and a clinical search for PD in the Phillipine Islands is currently being conducted by members of this laboratory.

The clinical and pathological characteristics of long surviving cases of Guamanian ALS, that is of more than ten years duration, are currently under study. Long surviving cases of ALS in Guam are younger, have a familial occurrence, have a different sex ratio, and show a different pattern of disease progression than those with a normal duration of disease.

Additional studies on HLA, dermatoglyphics and other gene markers, on osteoporosis and osteoarthritis, on heavy metals and other environmental toxins and on a ten-year follow-up study of the descriptive epidemiology of ALS and PD are close to completion. Further studies based on these data are in the planning stages or already underway.

Previous studies in our laboratory have shown that ALS and PD patients from Guam had diminished levels of cellular immunity as determined by diminished response to skin test antigens, lymphopenia, diminished number of 'T' cells, and decreased mitogenic response, than those of age- and sex-matched Guamanian controls. Further, ALS patients with HLA BW-35 had diminished cellular immunity and shorter mean duration of the disease. This association was found to a lesser degree among PD patients and no association was detected in the controls. Using C₁g binding techniques, Oldstone *et al.* have shown high frequency of immune complexes in the sera of ALS patients in the continental United States. There was evidence of immune complex deposition in some of the kidneys of the ALS patients. The nature of these immune complexes was not determined. Studies of hepatitis B in the South Pacific reveal that hepatitis B virus is endemic in most of the Pacific Islands. There is high prevalence of hepatitis B surface (HBsAg) antigenemia, and most of the population has either HBsAg or antibody to HBsAg. It is common to have found both HBsAg and anti-HBsAg in many individuals in the population. Since immune complexes are known to cause immunosuppression, we investigated the prevalence of HBsAg, anti-HBsAg, and the immune complexes

due to HBsAg and anti-HBsAg in the sera of ALS and PD patients from Guam and healthy controls. Additionally, we also tested sera for the presence of hepatitis A antibody. The data showed that ALS patients have lower levels of anti-HBsAg than PD patients or controls. There was no significant HBs antigenemia or immune complexes in ALS and PD patients and controls. Almost all sera tested had antibodies to hepatitis A. These studies show that HBsAg and anti-HBsAg complexes were not responsible for the immunosuppression observed. The lower rates of HBsAg in this population may be due to sampling of older individuals.

In other areas of Micronesia, human biological field and laboratory studies continue. Studies of chronic respiratory diseases indicate that 75% of the children under five years of age were found to have asthma, while over 50% of the adults over 40 years of age were affected by chronic bronchitis, often with an asthmatic component, and typical chronic obstructive airway disease occurred in almost one-third of the male population over 50 years of age. As a result, pulmonary airway diseases constitute the most important cause of morbidity and mortality in the Western Caroline Islands.

Since chronic inflammatory neurological disease is known to follow togavirus (arbovirus) encephalitis infections of humans in Europe and Asia, sera from more than twenty American patients with chronic epilepsy and inflammatory brain disease were examined by hemagglutination for all togaviruses known to cause encephalitis of humans in North America. None had antibodies. It seems unlikely that togavirus encephalitis is an important cause of chronic inflammatory brain disease in the United States.

A survey of togaviral antibodies in several Pacific populations confirmed earlier studies of the geographic distribution of several viruses. A possible correlation between susceptibility to Ross River Virus and one red cell Rh subtype was found in a population of Papua New Guinea. Plaque and microtiter tests have been developed for groups A and B togaviruses, and neutralization tests are being performed on selected sera.

Serum and CSF specimens from schizophrenic patients and age- and sex-matched controls were obtained from Doctors Torrey and Wineberger of St. Elizabeth's Hospital, Washington, D.C. and Constantine Sakkles of the University of Maryland Hospital, Baltimore. These specimens were tested for group A and group B arboviruses using the hemagglutination inhibition test. Viral antigens used in the test were Eastern and Western Encephalitis, St. Louis encephalitis, and California encephalitis. There was no significant association of arboviral antibodies to schizophrenia. In the light of recent reports by Tyrell et al. of detection of cytopathic agents from the CSF and some controls, attempts will be made to do similar studies with the CSF samples on hand.

The work on the development of animal models for the study of persistent infections has continued. A foamy virus of chimpanzees (Pan 1, also called foamy virus 6) was isolated in this laboratory over ten years ago. In the chimpanzee it appears to be a latent virus, and can at times be isolated from brain explants of healthy animals. The mechanism of viral latency has been impractical to examine, however, due to the expense and scarcity of the chimpanzee for experimental purposes. Therefore, experiments were conducted to adapt Pan 1 virus to a more convenient laboratory host, and after several preliminary studies, we succeeded in adapting the virus to the mouse. Using

kidney and spleen explants from mice-infected neonatally, infectious virus has been isolated up to one month following inoculation, viral antigen has been demonstrated in the explants, and serum CF antibody has been detected. However, in no animal has it been possible to detect infectious virus or viral antigen directly in the organs themselves. We are currently studying the possibility of viral persistence for up to a year following inoculation, and evaluating the mice for any signs of disease during their natural lifetime. Integration of viral genome in the host cells is also under investigation in collaboration with Dr. Chev Kidson in Australia.

The model of lysogenicity and of subviral genetically active macromolecular structures from the study of bacterial viruses and bacterial genetics supply ample imaginative framework for an expression of our ideas of possible pathogenic mechanisms for kuru and CJD in man. The unconventional viruses of the spongiform encephalopathies tax even our imagination in relation to molecular biology gained from these studies in bacteria.

For a now-disappearing disease, kuru, in a small primitive population to have brought us this far is ample reason for pursuing intensively the challenges offered by the still inexplicable high incidence and peculiar profusion of different neurological syndromes, pathologically distinct yet apparently related to each other, which have been discovered in the several small population enclaves we have investigated. Thus, the high incidence of ALS, ALS-PD on Guam and among a small population of people in West New Guinea, coupled with the high incidence of ALS on the Kii Peninsula of Japan, may indeed profusion of different neurological syndromes, pathologically distinct yet apparently related to each other, which have been discovered in the several small population enclaves we have investigated. Thus, the high incidence of ALS, ALS-PD on Guam and among a small population of people in West New Guinea, coupled with the high incidence of ALS on the Kuu Peninsula of Japan, may indeed offer the best opportunity of solving the problem of this sclerosing disease which in the United States has an incidence as high as that of multiple sclerosis.

The delineation of infection as the etiology of heredofamilial and presenile and senile dementias of man was made possible only through the concomitant studies on the neurobiology of population isolates. In this area we have been engrossed in the investigation of deaf-mutism, mental subnormality and other congenital central nervous system defects associated with endemic goiter in the Central Highlands of Western New Guinea, as well as patterns of delayed puberty, slow growth rates, and of early aging in isolated Melanesian groups. Ethnic drug abuse (particularly of kava), strange patterns of psychosexual development, pseudohermaphroditism, and culturally-determined responses to pain, and roots of aesthetic expression, have all been under study. Foci in primitive population isolates of familial periodic paralysis, progressive muscular dystrophy (both the pseudohypertrophic type of Duchenne and the non-pseudohypertrophic distal type), amyotrophic lateral sclerosis and Parkinsonism, are also being investigated. Genetic studies on human evolution led to the discovery of new genetic factors among haptoglobin, hemoglobin, and red cell enzyme pleomorphisms and the definition of their biochemical structure.

The further significance of scientific investigations of small population enclaves of remote populations was even more dramatically apparent during the 1975-1976 and the current 1979 field trip of the Chief of LCNSS, with his re-evaluation of what may turn out to be one of the largest "epidemics of

epilepsy" ever recorded. This continues to occur in the Wissel Lakes area of West New Guinea and is the result of cysticercosis, an infestation with the larvae of Taenia solium, the pig tapeworm, newly introduced into New Guinea. Our recent studies have led us to conclude that the natural history of cysticercosis epilepsy is not a result of death of the worm, scarring and calcification of lesions, as much of the literature suggests, but is an early sign of inflammation from new invasion of the brain by the Taenia larvae. Convulsions often occur even before the first subcutaneous nodules appear, and as the nodules increase in number, additional seizures occur. The high incidence of severe third-degree burns, which may even result in death, is a direct result of cysticercosis-induced seizures that occur during sleep, throwing the patient into the house fire. The unclothed people, living at a 2000 meter elevation, need to sleep close to the home fires on cold nights. We are able to date the first introduction of Taenia solium into the area and to plot the spread of taeniasis in pigs and man, and of cysticercosis and associated epilepsy in man, to other previously Taenia-free areas. During this year, we have learned that the cysticercosis has spread both in swine and man throughout the West New Guinea Highlands and is now in the Baliem region. With Dr. Budi Subianto, the local Indonesian medical officer, a visiting scientist in our laboratory, we have planned a neuroepidemiologic study aimed at elucidating the natural history of the epilepsy and acute psychoses and other neurological complications that have occurred concomitantly with the emergence of subcutaneous cysticercosis nodules.

Recently, we developed an enzyme-linked immunoabsorbent (ELISA) serological test for diagnosis and seroepidemiological surveillance of cerebral cysticercosis. Sera collected from adjacent populations prior to the introduction of T. solium and in 1974 and 1977 from patients with epileptic seizures, subcutaneous nodules, and other manifestations of cysticercosis at the Enarotoli hospital were studied. Positive control sera and cerebrospinal fluid (CSF) were from patients with neurocysticercosis in Mexico: their clinical disease had been previously confirmed by the presence of complement-fixing antibodies to cysticercus antigens. For the ELISA test cysticercus antigens were high speed supernatant of a sonicated 20% suspension of cysticercoi dissected from Balinese pigs killed in Jakarta; control antigens were similarly prepared from normal pig tissues. The ELISA procedure was that of Voller and Bidwell (1975) and Yolken et al. (1977) for rota virus assays. Titers were expressed as ratio of highest dilution of serum bound by cysticercus antigen to that bound by control antigen of same protein content. Standardization was done using antisera prepared in rabbits injected with cysticercus antigen in complete Freund's adjuvant. In symptomatic patients 5 of 6 (83%) with skin nodules, 7 of 9 (78%) with convulsions and skin nodules, and 7 of 16 (44%) new epileptics without skin nodules had antibody while among non-symptomatic residents of the Wissel Lakes area 4 of 52 (8%) had antibody. None of the 281 sera collected from people outside of the Wissel Lakes area had cysticercus antibody. Among the specimens from Mexican patients with neurocysticercosis 11 of 14 (79%) of the sera and 20 of 25 (80%) of CSF had antibody with geometric mean titers of 580 and 1600, respectively.

Higher percentage of positive patients with systemic cysticercosis may possibly be due to exposure to a larger antigenic mass. The lower positive rates observed among cerebral cysticercosis patients may be due to lack of antibody response due to direct massive infection of the brain by the parasite and short incubation period prior to detection of convulsions. The study shows

that the cysticercosis epilepsy epidemic in the Ekari people in West New Guinea was restricted to the Wissel Lakes area and no further spread was observed in the adjoining area. Importance of the cerebral cysticercosis in the third world countries cannot be underestimated. The ELISA test provides a simplified sensitive technique adaptable to field use for determining the presence and magnitude of human infections with cysticercus. However, because of its sensitivity cross reactivity has been observed to occur with antibodies to other parasitic diseases. This has led the studies on the development of techniques to produce purified cysticercosis antigens for enhancement of the specificity of the reactions.

As previously reported, the Chief of LCNSS was invited by the Soviet investigators to participate in the investigations in the U.S.S.R. of a unique degenerative disorder of the nervous system, Vilyuisk encephalitis. This disease occurs only in the Yakut region of Eastern Siberia and has many features of a slow virus disease. In August 1979 a field study was completed, the first by any western investigator, and many patients with VE were seen throughout the Iakut area. The diseases of Siberia and the last two decades of Soviet work on the disease, which is clearly infectious, were reviewed. We shall continue our collaborative study of this disease with our Soviet colleagues.

During the period covered by this report significant progress has been made on our studies begun in 1953 on the hemorrhagic fevers with renal syndrome that severely affected United Nations troops during the Korean War and for which an etiologic agent had not been isolated in spite of enormous efforts on the part of the Walter Reed Army Institute of Research of which we were then a part. The isolation by Lee and Lee in 1978 of the viruses responsible for HFRS has provided us the opportunity to reinvestigate this disease, characterize the virus and carry out collaborative studies with colleagues in China, the USSR, Finland, Sweden, Yugoslavia, Japan and Korea. In our first review of hemorrhagic fever with renal syndrome Gajdusek in 1956 indicated that clinical severity, particularly hemorrhagic manifestations, of this chronic viral nephropathy varies from one geographic region to another. Nephropathia epidemica (NE) of Scandinavia appears to be a mild form of HFRS or Korean hemorrhagic fever (KHF) with no or very minimal hemorrhagic manifestations. Mortality in the Far East (China, Korea, USSR) ranges from 5-30%, in European USSR it is lower, while NE is rarely fatal. The sylvatic reservoir for the virus in Scandinavia and European USSR is in wild voles (*Clethrionomys* sp.), whereas in Eastern Asia it is in the field mouse (*Apodemus agrarius*). The rat, *Rattus rattus*, appears to be the reservoir in Japan and in urban foci in Korea. Seasonal occurrence varies. Thus, cases are most frequent in the late fall and winter in Scandinavia at a time when wild voles enter dwellings and graineries. In southern and central China cases are more frequent in the autumn, during threshing season, and epidemiology has incriminated the respiratory route of infection. In both East and West sporadic cases occur yet epidemic outbreaks are frequent. This seems to be determined by the particular circumstances of exposure to the rodent reservoir. The military experiences in the Soviet Far East, Manchuria, and Korea of the Russian, Japanese, and United Nations armies, respectively, indicated two epidemic peaks, the first in late spring and early autumn, and the second in late summer and early fall; this was taken to suggest mite- or chigger-borne infection, as is the case with Tsutsugamushi disease. Lee, however, has not found virus in ectoparasites collected from infected rodents. The virulence, as evidenced by hemorrhagic manifestations, systemic reaction and mortality varies as one moves from Far Eastern Asia to eastern and

northern Europe. This parallels the shift of virulence of tick-borne encephalitis across the Eurasian land mass. However, Japanese cases are less severe, resembling NE more than KHF; possibly, the virus in rats is less virulent for man. Detailed serological comparisons of strains isolated in different regions are necessary to establish the closeness or divergence of the etiological viruses in various foci, and recent adaptations of the virus to laboratory rats, athymic nude mice, and tissue culture have now made this possible.

The first clear-cut evidence that hemorrhagic fever with renal syndrome virus infections were occurring by the respiratory route stems from the large outbreak of laboratory infections in Moscow in 1962 with 83 affected laboratory workers. A more recent epidemiological study of infections in medical research laboratories in Japan has indicated a respiratory route of infection of laboratory workers working in animal experimental rooms in contact with enzootically silently infected commercially reared white Wistar rats. Epidemiological studies in outbreaks in China (Xu et al., 1979) also led to the conclusion that most infection was by contaminated aerosols. Clinical and epidemiological studies in Scandinavia, Hungary, the Soviet Far East and Korea failed to directly incriminate the respiratory route of infection. But exposure to urine and feces contaminated foodstuffs and aerosols, or anthropod vectors, and ectoparasites such as mites and chiggers, on infected rodents were usually thought to be the source of human infection. However, it is now evident that infection occurs most often by the respiratory route from contaminated aerosols produced by the asymptotically infected reservoir rodents. Whether saliva and respiratory droplet infection--the only secretions from which virus has been isolated--is the only source of such aerosol contamination remains to be proved. Finally, high titer antigen has been found only in the lungs in infected wild mice (*Apodemus agrarius*), voles (*Clethrionomys glareolus*), wild urban rats (*Rattus rattus*), and laboratory rats of the Wistar strain in Japan; other tissues contain less concentrations of antigen as demonstrated by immunofluorescence. In experimentally infected white rats (Wistar and Fischer strains) and athymic nude mice the virus also appears in highest concentration in the lungs. The virus has to date been isolated only from lung, saliva, throat washings and blood of human patients, and no other tissue or secretion has yet been found to be infectious. In naturally and experimentally infected rodents the virus has not to date been isolated from feces or urine, but it has been obtained regularly from lung, saliva, and acute phase blood.

Until recently, the serological relationship between Scandinavian nephropathia-epidemica (NE) and Korean hemorrhagic fever (KHF) has been established (Svedmyr, 1978; Lahdevirta, 1979). This was first done using only as antigen KHF virus propagated in the lungs of naturally and experimentally infected *Apodemus agrarius* mice. We have recently confirmed this antigenic relationship by demonstrating specific neutralizing antibody to KHF virus in convalescent sera from patients with NE. Similar relationships have been shown for HFRS in European Russia with KHF virus. However, until the European virus was isolated from NE in Finland, it was previously impossible to check for immunological crossings in both directions. This has now been done and it is clear that NE sera react with Korean antigen in the immunofluorescent tests at almost the same titers with the homologous antigen from naturally infected or experimentally infected *Clethrionomys* lung. KHF human sera, on the other hand, give much higher titers with the homologous Korean virus in *Apodemus* lung than with the Finnish virus in *Clethrionomys* lung. Sera from patients convalescent

from HFRS in southern and central China react by immunofluorescence similarly to KHF sera as sera from HFRS patients in the Soviet Far East and in Japan. All these Asian sera (Chinese, Soviet, Korean and Japanese) from HFRS patients as well as Scandinavian NE sera neutralize several \log_{10} of KHF virus but qualitative cross neutralization tests have not yet been possible since the NE agent is only propagated with difficulty in *Clethrionomys voles*. Thus, the serological crossing is a partially one-way cross, with KHF sera reacting at 10- to 20-fold lower titer with NE antigen than with the homologous antigen, while, in contrast, Scandinavian NE sera show only a 2-fold reduction in titer with lung from *Apodemus* or nude mice infected with KHF than with the homologous antigen in *Clethrionomys* lung. Where in crossing Soviet Eurasia the shift to the NE from the KHF serological type occurs, remains to be determined. Sera from Balkan (Czechoslovakia, Hungary, Bulgaria, Rumania, and Yugoslavia) cases of HFRS have not yet been available for such study.

In a previous report (XIV Pacific Science Congress, 1979) we conjectured about the possible presence of unrecognized hemorrhagic fever with renal syndrome (HFRS) in North and South America and other areas of the world wherein the disease had not previously been recognized. The natural host of HFRS in northern and eastern Europe, *Clethrionomys* sp., is indigenous across northern North America in Canada and the United States from Maine to Alaska. The murine host of the viurs of Korean hemorrhagic fever (KHF), *Apodemus* sp., is not found in the Americas. *Clethrionomys*-borne disease in Europe has proved to be less severe clinically, and demonstrates fewer hemorrhagic symptoms than the *Apodemus*-borne disease in eastern Asia (China, USSR, and Korea). Thus, a milder form of nephropathy associated with little or no hemorrhagic diasthesis as in nephropathia epidemica (NE) in Scandinavia might be expected in the Americas. Using the indirect immunofluorescence test for demonstrating specific antigen-antibody reactions in KHF infections we have tested sera from Alaska, South America, and India. In the first 100 sera we studied from Alaska we reported no antibodies to KHF virus; however, when this series was extended to 600 specimens a single serum had specific antibody to KHF virus at titer 1:128. We also tested 4 cerebrospinal fluids (CSF) and 16 convalescent sera from children with an undiagnosed acute febrile illness in Santa Cruz, Bolivia. Although none of the 4 CSF reacted, 2 of the 16 sera had antibody titers to KHF virus of 1:256 and 1:128, respectively. Of 251 sera from residents of remote rural villages in India, 2 had antibodies to KHF virus; a 35-year old male gardener and a 27-year old female with titers of 1:256 and 1:640, respectively. Casals has found (personal communication) that high titering specific antibody to KHF virus failed to react in the HAI test against Japanese B, Murray Valley, Omsk hemorrhagic fever, and Chikungunya antigens. We found that high titering rabbit antisera or mouse ascitic fluids to more than 30 arboviruses, including Rift Valley fever and Junin viruses, and antisera to simian hemorrhagic fever virus did not react with KHF virus in the IF test. No other viruses are known to cross react with HFRS by the IF test. Neutralization tests on the few positive sera we have found from Alaska, Bolivia and India are in progress. These preliminary data suggest a possible wider distribution of HFRS viruses than is now known and further seroepidemiological screening from other parts of the world is clearly needed.

Continuing our more than three decades on work on the arthropod-borne viruses we have this year completed a study on human variation and infection with these viruses in humans in New Guinea. Antibodies to group A (Chikungunya, Getah, Sindbis, Ross River) and group B (dengue 2 and 4, Murray Valley

encephalitis, Japanese encephalitis, Yellow fever, Zika) arboviruses were measured by hemagglutination inhibition and neutralization in sera from selected aboriginal populations of New Guinea. Antibodies to Murray Valley encephalitis and Ross River viruses were highly prevalent in most of the lowland populations. For each population the presence of antibodies was correlated with 12 genetic polymorphic systems: 7 blood groups (ABO, MN, Ss, Rh, P, Kidd, Duffy), 3 red cell enzymes (acid phosphatase, 6-PGD, PGM), and 2 serum proteins (haptoglobin and immunoglobulin Gm).

There were no significant associations between any marker system and Murray Valley encephalitis virus infection. For one population, two blood group systems, Rh and Kidd, showed statistically significant associations with antibodies to the Ross River virus. Among individuals with the Rh phenotype R₁R₀ (CcDee), the relative risk of infection with Ross River virus was five times less than that for other members of the population. The relative risk of Ross River virus infection in individuals with Kidd phenotype Jka- was approximately three times less than that of the Jka+ individuals.

The reasons for those associations are unknown. Hypothetical explanations include differences in cell membranes of some Rh and Kidd phenotypes impeding attachment of virus, hereditary impairment of immune responses to the virus, shared antigens between the virus and blood-group substances resulting in immune tolerance, and decreased biting by mosquitoes of individuals with particular phenotypes. It is also possible that some genetically related social subgroup of people with less exposure to mosquitoes exists in the population. The associations between Rh and Kidd phenotypes and susceptibility to group A or other arthropod-borne infections must be confirmed by studies of larger populations living where such infections are endemic.

The development and maturation of the two major projects of this laboratory has resulted from cross-fertilization of each since their origin, and both have grown from the basic studies on child growth and development and disease patterns in primitive cultures. Although the two projects, each composed of many subsections, differ markedly in the questions they address and the techniques of investigation they employ, much of the field data collected from one project is also requisite for the studies in other projects. Both are served by the same investigators, who function as a team. These scientists derive their creative stimulus, dedication and enthusiasm to a great extent from the atypical and exotic biological, social and cultural materials presented, and the diverse, frequently unconventional, approaches of the two projects.

Principal Investigators: D. Carleton Gajdusek, M.D.
Clarence J. Gibbs, Jr., Ph.D.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 NS 01282-16 CNSS	
PERIOD COVERED October 1, 1979 through September 30, 1980					
TITLE OF PROJECT (80 characters or less) Neurobiology of Population Isolates: Study of Child Growth and Development, Behavior and Learning, and Disease Patterns in Primitive Cultures					
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PRINCIPAL INVESTIGATORS: D. Carleton Gajdusek, M.D., Chief, LCNSS; Clarence J. Gibbs, Jr., Ph.D., Deputy Chief, LCNSS; David M. Asher, M.D., Paul W. Brown, M.D., Ralph M. Garruto, Ph.D., and Lon R. White, M.D. Michael Alpers, M.D.; Richard Benfante, M.A.; Judith Farquhar; Peter Fetchko, M.A.; Dmitry Goldgaber; Chev Kidson, M.D.; Klaus Mannweiler, M.D.; Colin L. Masters, M.D.; Steven Ono; Robert G. Rohwer, Ph.D.; Donald Rubinstein, Ph.D.; Vincent Zigas, M.D. Jacques Bert, M.D.; Francoise Cathala, M.D.; Kwang-Ming Chen, M.D.; Louis Court, M.D.; Olivia Cruz, M.D.; Arwin R. Diwan, Ph.D.; Richard Feinberg, Ph.D.; Father David Gallus; Fusahiro Ikuta, M.D.; David E. Kohne, Ph.D.; Robert MacLennan, M.D.; Jesus Raglmar; John Runman; Frank Saul, Ph.D.; Wulf Schiefenhovel, M.D.; Koiye Tasa; Yushiro Uebayashi, M.D.					
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LAB/BRANCH Laboratory of Central Nervous System Studies, Intramural Research Program					
SECTION					
INSTITUTE AND LOCATION National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205					
TOTAL MANYEARS: 12		PROFESSIONAL: 8		OTHER: 4	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS					
SUMMARY OF WORK (200 words or less - underline keywords) Studies of human biology of vanishing primitive societies focus on neurological development and learning patterns in diverse cultural experiments in the human condition found in such isolated groups. Laboratory techniques of molecular biology, immunology, virology, endocrinology and biochemistry in these cultures and field epidemiological, genetic and clinical studies are aimed at problems more appropriately studied in small isolated primitive bands than in civilized societies. Data and specimens collected over years on expeditions to Micronesia, Polynesia, Solomon Islands, New Hebrides, New Guinea, Indonesia, S. America, Asia and Africa are used. Studies on nutrition, reproduction, fertility, neuroendocrine influences on age of sexual maturation and aging, genetic polymorphisms, genetic distance, unusual and odd employment of the higher cerebral CNS function of language learning, cognitive styles, computation (calculation without words or numbers) and culturally modified sexual behavior elucidate alternative forms of neurologic functioning for man which we would be unable to investigate once the natural cultural experiments in primitive human isolates were amalgamated into the cosmopolitan community of man. Foci of high incidence prevalence kuru, ALS/PD, epilepsy, other neurological degenerations, hysterical disorders, schizophrenia, neoplasms, goiter, cretinism, rheumatoid diseases, diabetes, asthma, chronic lung disease, malaria, filariasis, leprosy, cysticercosis and other infections are investigated.					

COOPERATING UNITS: continued

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VENEZUELA: L.T. Laffer and F. Melchiorri, Caracas.

- Sub-Project I: Study of the developmental patterning of the human nervous system (cybernetics of human development).
- Sub-Project II: Human evolutionary studies in isolated primitive groups.
- Sub-Project III: Studies of isolated Micronesian populations.
- Sub-Project IV: Studies of isolated New Guinea populations.
- Sub-Project V: Studies of Australian Aborigines.
- Sub-Project VI: Studies of isolated New Hebrides and Solomon Islands populations.
- Sub-Project VII: Studies of Central and South American Indians.
- Sub-Project VIII: Developmental, genetic and disease patterns in primitive populations of Asia, Africa, Indonesia, Melanesia, Micronesia, Polynesia and the Arctic.

- Sub-Project IX: Experimental developmental neuropsychiatrics in infantile programming: an empirical approach to the language of information input into the nervous system.
- Sub-Project X: Ciphers and notation for the coding of sensory data for neurological information processing.
- Sub-Project XI: Racial distribution and neuroanatomic variations in the structure of the human brain.
- Sub-Project XII: Studies of high incidence of neurological diseases in specific racial and ethnic groups and in primitive or geographic population studies.

Project Description: Neurobiology of Population Isolates: Study of Child Growth and Development, Behavior and Learning, and Disease Patterns in Primitive Cultures (described fully on pages 1-IRP/LCNSS through 22-IRP/LCNSS).

Publications: Listed on pages 35-IRP/LCNSS through 41-IRP/LCNSS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 00969-16 CNSS
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Chronic CNS Disease Studies: Slow, Latent and Temperate Virus Infections		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PRINCIPAL INVESTIGATORS: D. Carleton Gajdusek, M.D., Chief, LCNSS; and Clarence J. Gibbs, Jr., Ph.D., Deputy Chief, LCNSS Herbert L. Amyx, D.V.M.; Tomonobu Aoki, M.D.; David M. Asher, M.D.; Sina Bahmanyar, M.D.; Maria-Teresa Borrás, Ph.D.; Paul W. Brown, M.D.; Ralph M. Garruto, Ph.D.; Patrick Gourmelon, M.D.; David T. Kingsbury, Ph.D.; Yasuo Kuroda, Ph.D.; Pyung-Woo Lee, Ph.D.; Maryellen F. Masciangelo, Ph.D.; Colin L. Masters, M.D.; Shigeru Mori, M.D.; Seiho Nagafuchi, M.D.; Robert G. Rohwer, Ph.D.; Lon R. White, M.D.; Richard T. Yanagihara, M.D. Francoise Cathala, M.D.; Louis Court, M.D.; Philippe de Micco, M.D.; Arwin R. Diwan, Ph.D.; Sergio Galvez, M.D.; Dmitry Goldgaber; Jaap Goudsmit, M.D.; Chev Kidson, M.D.; Takao Makifuchi, M.D.; Klaus Mannweiler, M.D.; Marie-Claude Moreau-Dubois, Ph.D.; Ryoichi Mori, Ph.D.; Arne V. Svedmyr, M.D.		
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LAB/BRANCH Laboratory of Central Nervous System Studies, Intramural Research Program		
SECTION		
INSTITUTE AND LOCATION National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205		
TOTAL MANYEARS: 24	PROFESSIONAL: 14	OTHER: 10
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Studies elucidate cause and patho- genesis of chronic degenerative CNS disorders with emphasis on MS, ALS, parkin- ism-dementia, Parkinson's, Pick's, and Alzheimer's diseases, Huntington's chorea, supranuclear palsy, other presenile dementias, chronic encephalitis with focal epilepsy, muscular dystrophies, chronic schizophrenia, SSPE, PML, dialysis encephalopathy, and intracranial neoplasms. Even familial, apparently hereditary diseases may be slow virus infections. Subacute spongiform virus encephalopathies (kuru and Creutzfeldt-Jakob (CJD) diseases of man; scrapie and mink encephalopathy) are caused by unconventional viruses with unique properties posing important theoretical problems to microbiology and molecular biology; a major goal is elucidation of their structure and mechanisms of replication. Transmissible virus dementias are increasingly recognized worldwide causes of death: high incidence foci, transmission by corneal transplant or brain surgery, and occupational hazards from exposure to brain occur. In order to determine the usual mode of infection with the virus, a worldwide epidemiological study of transmissible virus dementia (CJD) cases is underway with special attention to familial clusters of cases and with a quest for possible relationship of scrapie of sheep to the human disease.		

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YUGOSLAVIA: H. Vesenjak-Hirjan, M.D., Department of Virology, Medical Faculty, University of Zagreb, Zagreb.

- Sub-Project I: Attempts to isolate, identify and characterize transmissible agents from humans and animals with subacute degenerative diseases of the central nervous system: transmissible hereditary diseases, presenile and senile dementias of the sporadic and familial types and primary sclerosing and demyelinating diseases.
- Sub-Project II: Characterization and pathogenesis of kuru virus.
- Sub-Project III: Characterization and pathogenesis of Creutzfeldt-Jakob disease (transmissible dementia) virus.
- Sub-Project IV: Scrapie: studies on the purification, physical and biological characterization and nature of the virus.
- Sub-Project V: In vitro cultivation of the viruses of the subacute spongiform virus encephalopathies in cell cultures.
- Sub-Project VI: Host range of susceptible laboratory animals to the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project VII: Strain variations among the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project VIII: Cell-fusing properties of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project IX: Resistance to radiation of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project X: Resistance to disinfectants of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project XI: Tissue and cell culture techniques used to unmask slow infections of man and animals using brain and viscera biopsy and early autopsy, bone marrow and peripheral leucocyte specimens.

- Sub-Project XII: The syncytium-forming viruses (simian and human foamy viruses).
- Sub-Project XIII: Studies on transformed human brain tissue in vitro and characterization of associated virus.
- Sub-Project XIV: Electron microscopic membrane studies of subacute spongiform virus encephalopathies.
- Sub-Project XV: Characterization and identification of new herpes viruses from explant cultures of tissues from subhuman primates.
- Sub-Project XVI: Studies on persistent asymptomatic cytomegalovirus infections of healthy rhesus monkeys.
- Sub-Project XVII: Focal movement disorders in rhesus monkeys following experimental infection with a strain of tick-borne encephalitis virus.
- Sub-Project XVIII: Fluorescent antibody studies on the intracellular localization and identification of viral antigens in vivo and in vitro in tissues from patients with subacute diseases of the central nervous system.
- Sub-Project XIX: Isolation and characterization of adenovirus from the urine of chimpanzees.
- Sub-Project XX: Development of serological and immunological test system for use in the study of slow infections of the central nervous system.
- Sub-Project XXI: Immune responsiveness of multiple sclerosis patients to established viral antigens by detection of specific antibodies in serum and cerebrospinal fluids collected serially during remission and exacerbation.
- Sub-Project XXII: Animal management and intercurrent diseases in subhuman primates on long-term studies of slow infections.
- Sub-Project XXIII: Studies to determine the possible presence of cryptic viral genomes in human brain tissues.
- Sub-Project XXIV: Sequential development of kuru-induced neuropathological lesions in spider monkeys.
- Sub-Project XXV: Studies on the isolation, characterization, identification and pathogenicity of type C viruses from human and animal tissues.
- Sub-Project XXVI: Biochemical studies of the etiology of amyotrophic lateral sclerosis and parkinsonism-dementia.

- Sub-Project XXVII: Study of mitochondrial mutants from scrapie-infected mouse brain cells.
- Sub-Project XXVIII: Isolation and characterization of the etiological agent of Scandinavian nephro-nephritis epidemica.
- Sub-Project XXIX: The pathogenesis of Korean hemorrhagic fever virus and the elucidation of its biological and physical properties.
- Sub-Project XXX: Worldwide seroepidemiological evidence of antibodies in human populations to the virus of Korean hemorrhagic fever.
- Sub-Project XXXI: Development of an enzyme-linked immunoabsorbent (ELISA) test for the diagnosis and epidemiology of cysticercosis-induced epilepsy.
- Sub-Project XXXII: Studies on the cytochemical and morphological properties of neurons cultured in vitro.
- Sub-Project XXXIII: Development of immunological markers for the detection of autoantibodies to neurofilaments in the sera of patients with subacute spongiform encephalopathies.
- Sub-Project XXXIV: Studies to determine the neurophysiological changes of neurons in vitro infected with CJD.
- Sub-Project XXXV: Effects of the subacute spongiform viruses on nerve cells grown in vitro.
- Sub-Project XXXVI: In vivo and in vitro studies to determine the etiology of myasthenia gravis.
- Sub-Project XXXVII: Neurophysiological study of animals experimentally infected with subacute spongiform virus encephalopathies.

Project Description: Chronic Central Nervous System Disease Studies
(described fully on pages 1-IRP/LCNSS through 22-IRP/LCNSS)

The projects (I through XXXVII) listed herein, as itemized in the Project Reports of previous years, have continued throughout this year and have been expanded, as are reflected in the extensive list of publications and the summary in pages 1-IRP/LCNSS through 22-IRP/LCNSS. Contractural phases of this work are being conducted at: Gulf South Research Institute, New Iberia, Louisiana; and Public Health Research Institute of the City of New York, Inc., Otisville, New York.

Publications: Listed on pages 35-IRP/LCNSS through 41-IRP/LCNSS.

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CONTRACTS

Laboratory of Central Nervous System Studies, NINCDS

October 1, 1979 through September 30, 1980

Gulf South Research Institute
New Iberia, Louisiana

Contract #N01-NS-*-09931

\$ 600,000.00

Public Health Research Institute of the City of New York, Inc.
Otisville, New York

Contract #N01-NS-7-0082

\$ 120,000.00

Litton Bionetics, Inc.
(Administration by NCI)

Contract #N01-C0-75380

\$ 385,700.00

Mrs. Elisabeth Beck
Institute of Psychiatry
London, England

Contract #263-78-C-0049

\$ 24,500.00

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Clinical Neurosciences Branch
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT

October 1, 1979 through September 30, 1980
Clinical Neurosciences Branch
National Institute of Neurological and Communicative
Disorders and Stroke

Susumu Sato, M.D., Acting Chief

Summary of Program Activity

As of August 1, 1979, the Applied Research in the Surgical Neurology Section, CNB, was transferred to the Surgical Neurology Branch. The Branch activity consists of research and clinical diagnostic service, that involves a total of 3.9 man/year (1 professional and 2.9 technical-clerical).

I. Clinical Diagnostic Service:

During this reporting period, a total of 1066 electroencephalograms were obtained in patients who were referred to our Branch as part of their routine clinical investigation or for specific research projects from other Branches of our Institute or from other Institutes.

The distribution of these referrals according to the Institute of origin is as follows:

<u>Institute</u>	<u>No.</u>	<u>%</u>
NINCDS	609	57.1
(OPD)	(287)	(26.9)
NIMH	145	13.6
NICHD	71	6.7
NCI	64	6.0
NHLBI	44	4.1
NIAID	37	3.5
NIAMDD	30	2.8
OPD (others)	51	4.8
MISC.	15	1.4
	1066	100.0

There is a slight increase in the total number of electroencephalograms as compared with that of the previous year. About 40% of the EEG requests came from Institutes other than NINCDS. The service continues to supply useful information for several research projects in our Branch which

collaborate closely with other units, especially the section on Clinical Epilepsy of the Experimental Therapeutic Branch. Miscellaneous category included the bedside EEG recordings in the CCU and electrocorticography in the operating room.

In the end of 1979, the Branch obtained a signal averager (Nicolet CA 1000) and has provided a diagnostic service of cerebral evoked responses to the Institute. The Branch is now able to provide visual, auditory, and somatosensory evoked response testing and has done testings in over 120 patients. Requests for the cerebral evoked potentials has been increasing.

The Branch also provides material for the training in Clinical Electroencephalography so that each year one or two Clinical Associates become eligible for the American Board of Qualification in EEG.

II. Research Activity:

There are seven projects which are active during this reported period. Five of them are continued from the previous years. Two projects needed a revision of the title, because of some changes in methodology. Two projects are new.

a) Clinical

The analysis of clinical seizure patterns in different forms of epilepsy continues to be a main field of interest for this Branch. The Branch is in the process of obtaining an EEG machine with video monitoring system which allows us to observe ictal, clinical and EEG patterns simultaneously and which also gives us an opportunity to record them. This will tremendously increase the chances of electro-clinical correlation.

Electroencephalographic manifestations were studied in 10 patients (6 males and 3 females, 12 to 56 years) with gyrate atrophy of the choroid and retina which is an autosomal recessive multi-system disorder, associated with an absence of ornithine-aminotransferase resulting in an elevation of plasma and cerebral spinal fluid ornithine levels. The EEG findings included intermittent theta or sharp wave activity of unilateral or bilateral distribution and rhythmic theta activity in the left temporal areas, posterior and occipital area, central area or diffusely. These findings suggest that EEG changes are indicative of primary CNS involvement in gyrate atrophy of the choroid and retina.

Electroencephalographic changes also were studied in 16 patients (1 to 16 years) with nephropathic cystinosis which is an autosomal recessive disorder associated with accumulation of free cystine in the lysosomes of many tissues. The effects of cysteamine which lowers intracellular cystine content also was studied. Thirteen patients had normal pre-treatment EEGs. Two patients had minimal disturbances of background activity and one patient had a diffuse and focal sharp and slow wave complexes prior to

treatment. During therapy of cysteamine, two patients experienced obtundation and changes in the background rhythms, four patients had paroxysmal diffuse sharp and slow wave complexes and one patient had rhythmic posterior slow activity. No patients had seizures at any time. Thus, cysteamine can produce a mild dose dependent EEG change compatible with encephalopathic as well as paroxysmal abnormalities.

In the end of 1979, the Branch obtained a Nicolet CA 1000 with the necessary accessories and since then, the Branch has begun the investigation of cerebral evoked potentials (visual, auditory and somatosensory). The aim of this research is to establish the practicability and diagnostic values of particular modality of tests in various neurological disorders. For visual potentials photic flash was initially used but has been found to produce a significant diversity of results intra-individually as well as inter-individually, making comparison and interpretation difficult. Therefore, the main mode of visual stimulation has been changed to a reversing checkerboard pattern generated by the model NIC 1005 or projector pattern generator. Thereafter the visual evoked responses (VEP) have gained consistency and became interpretable. We are in the process of collecting normative data (latency, amplitude, and morphology) and some of the results are tabulated in the project description. These values (latency) are found compatible to those in literature. The VEP (latency measurement) has been found to be a sensitive test (prolonged latencies) to find a manifest or occult abnormality in the visual pathway in patients with Multiple Sclerosis. However, we have found that pattern VEP requires subjects' cooperation and constant attention, whereas, flash VEP can be used for uncooperative or unconscious patients. The Branch also initiated research on brainstem auditory evoked potentials to evaluate its diagnostic applicability. This test can be used to evaluate not only the auditory pathway but also the brainstem function. The latter aspect of correlation has just begun in this field of research and has been of our major interest. We also are in the process of collecting normative data (latency, amplitude, and morphology) and some of the results are tabulated in the project description. Prolonged latencies and distorted morphology have been noted in MS patients. Our future plan is to conduct research on somatosensory evoked potentials. Although its literature is large, there has been no consistent technique or results in this mode of evoked potentials.

The neuropsychological research program actively studied cognitive and emotional changes accompanying neuropsychiatric diseases, including Huntington's (HD) and Alzheimer's Disease. In addition, the study extended the search for behavioral clues to the presence of the HD gene, and developed a wide range of neuropsychological tests to evaluate individuals classified "at-risk" for HD. While testing the basic assumption that structural-functional defects appear in the neo-striatal system during HD, the investigation also examined whether these dysfunctions were associated with an asymmetry of involvement with left or right brain mechanisms.

The findings confirmed the presence of widespread cognitive and emotional impairment in individuals with Huntington's Disease, emerging as deficits in perception and memory, in solving visuospatial integrative tasks and in

utilizing spatial-directional clues. Moreover, patients with early stage symptoms seemed to be troubled in perceiving and encoding a variety of sensory messages presented via visual, auditory and tactile channels.

Clinically, it seems that frontal lobe-like deficits impair the patient's ability to plan, organize and schedule activities and to remember events, and as a result, lead to maladaptive and inappropriate reactions to environmental nuances. In the emotional section, the HD patients showed abnormal profiles on the Minnesota Multiphasic Personality Inventory (MMPI), with schizophrenia and depression being identified as benchmarks of the aberrant process.

As a group the at-risk subjects also showed a distinct pattern of weaknesses on several neuropsychological tests in comparison with matched normal subjects. The at-risk group did poorly with tasks demanding perceptual motor discrimination and learning. These preliminary findings raise questions about frontal lobe integrity in affected but pre-symptomatic at-risk subjects. The present study will be continued and incorporate biochemical and neuroradiographic parameters and data in an effort to present a statistical profile to identify individuals within the at-risk group who project a high probability for incurring HD.

Recently, a broad-based and integrated neuropsychological study was undertaken to identify emotional and cognitive defects in neuropsychiatric, dementia deteriorative processes. The study was designed to examine the nature and content of the specific amnesic deficit embedded in Alzheimer's Disease, and also to establish whether other cognitive processes are severely modified, specifically, perception, discrimination and encoding. The deficits of dementia expressed through disorientation, confabulation and other aberrant functions, contaminate the study of memory disabilities as it becomes important for rehabilitate and treatment purposes to clearly identify the exact nature and severity of functional defects.

Estimates of intellectual, memory and personality functions will be correlated with biochemical, radiographic and indices of dementia. In addition, electrophysiologic correlates are being established to study how patients perceive, learn and respond to a variety of sensory impressions in an effort to better understand the general breakdown in neuro-sensory processing demonstrated in Alzheimer's and other neuropsychiatric disorders.

b) Experimental Research:

In early 1980 the Branch began research in experimental epilepsy utilizing a Kindling model. Kindled seizures are produced by daily electrical stimulation of amygdaloid complexes in rat. So far, one rat has been fully kindled having clonic seizures in which the rat masticates, stands on hind limbs with a clonic movement of the face and fore limbs and shows a tendency to fall toward the non-stimulated side. The whole sequence lasts about 1 minute. Two more rats began to show electrographic after-discharges after each stimulation. We are in the process of establishing a standard method and technique to kindle rats and then our plan is to study interictal epileptiform discharge patterns in relation to ictal events, effective sleep-wake cycle in maturation on the epileptiform discharges. Following the above baseline investigation, we will study the biochemistry of Kindling process. The goals for this study are to determine the relationship of cerebral metabolism as it relates to the onset propagation and termination of the seizure process, to determine if there are any changes in GABA metabolism, to determine if any significant changes may occur in the cyclic nucleotides.

Other Activities, Honors etc.

In recognition of outstanding contributions to neuropsychological research, Dr. Paul Fedio was nominated as a "Fellow" in the American Psychological Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 00200-26-CN
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Cognitive and Emotional Profile of Neuropsychiatric Disorders. Former Title: Involuntary Movements		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: OTHER:	P. Fedio A. Martin P. Brouwers T. Chase C. Cox J. Bravo	Psychologist CN NINCDS Psychologist CN NINCDS Psychologist CN NINCDS Neurologist ETB NINCDS Psychologist CN NINCDS Psychologist CN NINCDS
COOPERATING UNITS (if any) Experimental Therapeutics Branch, NINCDS		
LAB/BRANCH Clinical Neurosciences		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.2	OTHER: 0.5
CHECK APPROPRIATE BOX(ES)		
<input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p> An <u>emotional</u> and <u>cognitive</u> profile was developed for individuals with <u>Alzheimer's Disease</u>, <u>Huntington's Disease</u> and those classified as "<u>at risk</u>" for <u>Huntington's Disease</u> from comprehensive <u>neuropsychological</u> assessment. The evaluation extended into <u>memory</u>, <u>learning</u> and <u>perceptual</u> areas, and included <u>personality</u> and emotional measures, utilizing standard and experimental tasks. These behavioral data will be collated with biochemical and neuroradiologic measures and indicators of deterioration and <u>dementia</u> will be developed. </p>		

Project Description:

Objectives:

To develop a comprehensive neuropsychological study of individuals presented with various neuropsychiatric disorders including Alzheimer's Disease, Huntington's Disease and offspring of parents with Huntington's Disease classified as 'at risk'. The project contains a threefold purpose: 1) Evaluate attentional, perceptual, memory and behavioral/emotional defects associated with neuropsychiatric disorders; 2) provide an objective assessment of cognitive emotional dimensions for individuals who are classified as 'risk' candidates for Huntington's Disease; 3) to establish a reliable diagnostic technique of being able to determine whether an 'at risk' individual may be presented with Huntington's Disease.

Methods Employed:

An integrated neuropsychological test battery, comprised of standard and experimental procedures was developed. The project included personality assessment for anxiety and psychosomatic traits and affective balance. In cognitive areas, standard psychometric test of memory and intelligence were used, supplemented by laboratory measures of perception, attention, spatial orientation, learning/memory and adaptive behavior. In addition, specialized techniques creating interhemispherical competition also formed an integral part of the test battery to evaluate neuropsychological deficits in patients with Huntington's and Alzheimer's Disease.

Major Findings:

The findings were consonant with emerging neurobehavioral research, describing widespread cognitive and emotive impairment in individuals with Huntington's Disease. These patients showed pervasive deficits in perception and memory, in solving visual spatial integrative tasks and in utilizing spatial directional clues. HD patients with mild symptoms experienced much difficulty in perceiving and encoding visual, auditory and tactile stimuli.

Interhemispherical tests suggested that during the early stages of Huntington's Disease, the patient may experience severe disruption to perceptual mechanisms which impact adaptive strategies and memory, and lead to inappropriate behavioral reactions. These data suggest that the initial symptoms may reflect defective callosal transmission and structural-functional changes within the fronto-striatal system, bilaterally. Moreover, the HD profile included deficits in grammatical composition, reduced word fluency, articulatory weaknesses, and difficulty in dealing with competing stimuli, functions which depend upon the integrity of frontal mechanisms. Clinically, these patients complained about an inability to plan, organize and schedule activities and to remember.

In addition to cognitive deficits, HD patients also manifested

emotional and personality changes which were reflected with abnormal scores on the Minnesota Multiphasic Personality Inventory (MMPI) and other selected tests, schizophrenia and depression being identified as benchmarks. The HD patients showed unusually high overt anxiety, and admitted concern about suicidal rumination and a wide range of personal-social difficulties.

As a group, the 'at risk' subjects showed selective cognitive weaknesses on several neuropsychological tests. Inspection of the findings indicated that the 'at risk' group did poorly in several areas of perceptual motor discrimination and learning, including perceptual desembedding, learning spatial paths, and formulating directional-spatial judgements. Further analysis of the interhemispherical tests support the preliminary findings and raise questions whether frontal lobe integrity is affected in pre-symptomatic, 'at-risk' subjects.

In personality spheres the 'at-risk' group did not exhibit significant psychopathologic disturbances. However, as a group the subjects were classified as dependent, introverted and compulsive, and exercise much concern about regulating anger. The study suggests that the risk for psychological maladaptive behavior appears to be increased for offspring of HD parents, and this needs to be addressed clinically and independently of the likelihood of inheriting HD.

Significance to Biomedical Research and the Program of the Institute: In view of the cognitive and emotional disabilities associated with neuropsychiatric disorders as Alzheimer's and Huntington's Disease, the project represents an empirical approach to identify neuropsychological profiles which are common and distinct to each disease process. These observations will be collated with neuroradiographic and biochemical data in an effort to develop a better understanding of the deteriorative impact in functional and neurologic sectors.

Proposed Course of the Project: The major study of the Huntington's subjects has been completed but new neuropsychological procedures are being developed to monitor progressive changes, time-reference to the disease process. In addition, a large series of Alzheimer's patients have recently been studied and the investigation should be completed within the next several months. The proposal intends to statistically portray a neuropsychological profile of dementia, including specific alteration in mood and cognition that are distinctly and uniquely associated with Alzheimer's or Huntington's Disease.

Publication: Fedio, P., Cox, C.S., Neophytides, A., Canal-Frederick, G., and Chase, T.N.: Neuropsychological profile of Huntington's Disease: patients and those at risk. In Chase, T.N., Wexler, N.S. and Barbeau, A. (Eds.): Huntington's Disease. Advances in Neurology, 23, New York, Raven Press, 1979, pp. 239-255.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01245 -15-CN															
PERIOD COVERED October 1, 1979 to September 30, 1980																	
TITLE OF PROJECT (80 characters or less) EEG Learning Correlates Using Scalp and Intracranial Depth Electrodes																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">P. Fedio</td> <td style="width: 20%;">Psychologist</td> <td style="width: 10%;">CN</td> <td style="width: 10%;">NINCDS</td> </tr> <tr> <td></td> <td>L. Mononan</td> <td>Psychologist</td> <td>CN</td> <td>NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>M. Buchsbaum</td> <td>Research Medical Officer</td> <td>AP</td> <td>NIMH</td> </tr> </table>			PI:	P. Fedio	Psychologist	CN	NINCDS		L. Mononan	Psychologist	CN	NINCDS	OTHER:	M. Buchsbaum	Research Medical Officer	AP	NIMH
PI:	P. Fedio	Psychologist	CN	NINCDS													
	L. Mononan	Psychologist	CN	NINCDS													
OTHER:	M. Buchsbaum	Research Medical Officer	AP	NIMH													
COOPERATING UNITS (if any) Technical Development, NINCDS Adult Psychiatry, NIMH																	
LAB/BRANCH Clinical Neurosciences																	
SECTION Office of the Chief																	
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																	
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.2	OTHER: 0.2															
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS																	
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Central processing</u> of information by the human brain was monitored and quantified by averaged <u>evoked response</u> techniques. The electrographic recording of left and right brain activity during <u>perception</u> in normal subjects was compared with that of patients with <u>neuropsychiatric disorders</u> (Alzheimer's). Suspect disturbances in <u>brain-behavior</u> relations in psychiatric patients were also evaluated, relating <u>left brain</u> dysfunction to <u>ideational disorders</u>, <u>right brain</u> to <u>emotional problems</u>. </p>																	

Project Description:

Objectives:

Evoked response measures were included as part of the broad-based investigation of altered neuropsychological function in patients with Alzheimer's disease in an attempt to assess brain integrity function during perception of auditory and visual information.

Methods Employed:

A series of attentional tasks, designed to evaluate brain processes, were used. Electroencephalographic (EEG) activity was recorded and averaged in evoked responses from scalp electrodes positioned symmetrically at temporal-parietal regions of the left and right hemispheres. Included for study were neurosurgical patients who had undergone unilateral removal of the temporal lobe, and Alzheimer's patients.

Major Findings:

All electrographic test runs were conducted off-line, and the evoked potential data are currently being processed. The study is in progress for the temporal epileptic patients. Preliminary observations with the Alzheimer patients reveal anomalies in EEG frequency/amplitude, corresponding to changes in attention and perception.

Significance to Biomedical Research and the Program of the Institute:

Behavioral data available from epileptic patients following unilateral temporal lobectomy reveal significant perceptual and learning deficits which are related to the laterality of surgery and to the specific character of the material. The technique employed in this project affords a more precise method for outlining cortical and subcortical systems in the human brain which mediate learning and memory. The research also provides physiologic and behavioral data for the comparison of neurologic and psychiatric patients in order to identify possible brain dysfunctioning in neuropsychiatric disorders.

Proposed Course of the Project: A computer has been acquired, and programs will be developed to provide off-line analysis of data. Specialized neuropsychological tasks will also be developed, and applied in the study of patients with neuropsychiatric disorders.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01424-14-CN																									
PERIOD COVERED October 1, 1979 to September 30, 1980																											
TITLE OF PROJECT (80 characters or less) Response Modulation by the Limbic System in Man: Neuropsychological and Physiological Changes																											
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COOPERATING UNITS (if any) None																											
LAB/BRANCH Clinical Neurosciences																											
SECTION Office of the Chief																											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205																											
TOTAL MANYEARS: 1.1	PROFESSIONAL: 0.6	OTHER: 0.5																									
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS																											
SUMMARY OF WORK (200 words or less - underline keywords) Emotional and sensory characteristics are studied in patients with <u>temporal lobe epileptic foci</u> . Patients and raters independently complete <u>true-false questionnaires</u> which probe specific behavioral and emotional traits, and permit analysis of distortions in self perception. In a separate investigation, patients rate various <u>emotions</u> displayed by <u>facial expressions</u> , and learn words with different emotional connotations. Temporal epileptic patients are compared with matched normal subjects and patients with other neurologic illnesses. Patients with a right temporal focus are compared with left temporal epileptics. Statistical analyses are employed to codify behavioral and sensory profiles of right and left temporal epileptic subjects. The research examines the role of the anterior temporal lobe in establishing <u>limbic associations</u> and differences between the <u>left</u> and <u>right hemispheres</u> in regulating emotions and sensory experiences in man.																											

Project Description:

Objectives:

1. To identify cognitive profiles of epileptics who have undergone unilateral temporal lobectomy, for the relief of intractable seizures; to evaluate relationships between seizure patterns and frequency, and change cognitive and behavioral parameters.
2. To evaluate the role of the temporal lobe in 'emotional preception and learning'. A procedure was developed to assess how accurately temporal lobe patients interpret various emotional states, and whether there may be a selective memory deficit for emotionally laden or neutral stimuli.

Methods Employed:

Specialized neuropsychological techniques and procedures are being designed and evaluated, and will include tests to identify attentional, perceptual and memory and communicative processes, the role of language encoding to facilitate recall, the adaptive strategies developed and utilized by brain-injured patients.

Major Findings:

The study is being redesigned and has not yielded patient data. New procedures and techniques are being developed and will be examined by a pilot study before actual testing with neurologic patients.

Significance to Biomedical Research and the Program of the Institute:
By identifying specific behavioral sequelae of a temporal lobe focus, these observations further neuroanatomical understanding of emotional processes. The results may be interpreted as a consequence of enhanced sensory-limbic associations. This interpretation regarding the effects of temporal lobe epilepsy in human subjects is consistent with extensive animal experimentation on sensory-limbic disconnections. The findings quantitatively support an asymmetry of emotional processing within the right and left hemisphere of man.

Proposed Course of the Project: Testing of additional psychiatric and neurologic contrast groups (nontemporal epileptics) is planned.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01658-13-CN										
PERIOD COVERED October 1, 1979 to September 30, 1980												
TITLE OF PROJECT (80 characters or less) Hemispheric Development and Specialization of the Intellectual Functions												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 30%;">P. Fedio</td> <td style="width: 25%;">Psychologist</td> <td style="width: 10%;">CN</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>C. Cox</td> <td>Psychologist</td> <td>CN</td> <td>NINCDS</td> </tr> </table>			PI:	P. Fedio	Psychologist	CN	NINCDS	OTHER:	C. Cox	Psychologist	CN	NINCDS
PI:	P. Fedio	Psychologist	CN	NINCDS								
OTHER:	C. Cox	Psychologist	CN	NINCDS								
COOPERATING UNITS (if any) None												
LAB/BRANCH Clinical Neurosciences												
SECTION Office of the Chief												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205												
TOTAL MANYEARS: <div style="text-align: center;">1.6</div>	PROFESSIONAL: <div style="text-align: center;">0.6</div>	OTHER: <div style="text-align: center;">1.0</div>										
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SUMMARY OF WRK (200 words or less - underline keywords) <p> The disabling effects of <u>brain damage</u> in man were evaluated with a broad range of <u>neuropsychological tests</u> evaluating <u>perception</u>, <u>learning</u> and <u>memory</u>. Changes in the intellectual behavior of neurologically impaired individuals were evaluated before and after <u>brain surgery</u>, and during <u>electrical stimulation</u> of the <u>cortical</u> surface and <u>subcortical</u> depths in the <u>thalamus</u> and <u>cingulum</u> of the brain. </p>												

Project Description:

Objectives:

1. Outline cortical and subcortical mechanisms which subserve cognitive functions and code information to be held for immediate (short-term) or for delayed (long-term) memory; compare the effects of injury to different brain regions, and develop an integrated model of the human brain for communicative skills and memory.

2. Examine the role of temporal lobe mechanisms in guiding visual behavior under altered or distorted perceptual conditions.

Methods Employed:

1. The laterality and general outline of cortical and subcortical zones instrumental in basic cognitive functions were mapped by electrical stimulation. A. verbal and nonverbal test, each with a perceptual and memory command were employed, and utilized photographs of commonplace objects or patterns with instructions to name or discriminate, and to remember each stimulus object or design.

Major Findings:

Brief electrical stimulation within the left temporal parietal cortex produced transient dysphasia, speech errors and an independent retrograde memory loss for verbal memoranda. In contrast, comparable electrical stimulation of homologous sites within the right cortex did not impair speech or verbal behavior. Instead, the patients experienced difficulty in matching and remembering complex visual patterns. Stimulation of the foot of the third frontal cortex (Broca's area) was associated with fewer naming errors, while stimulation of the anterior left temporal cortex rarely elicited difficulty in naming. It should also be noted that cortical speech mapping did not elicit patient reports of experiential or personal memories like those previously described by Drs. Penfield and Roberts at the Montreal Neurologic Institute.

Stimulation of the right brain produced a functional distinction between the anterior and posterior regions. As with naming errors and left brain stimulation, the occurrence of visual perceptual errors was coincident with stimulation of the right posterior, but not the right anterior surface. Similarly, errors of omission were the most prevalent on the discrimination problem.

Analysis of the memory errors yielded anatomically distinct zones for error classes. Specifically, anterograde errors were more prevalent with anterior temporal stimulation while retrograde errors were associated with stimulation of the posterior temporal parietal region. Functionally, recent information could be retrieved but newly perceived material could not be stored adequately during stimulation of the anterior cortical surface. In contrast, retrograde errors were seen

with posterior stimulation -- recently stored information could not be retrieved even though new information could be stored simultaneously. A comparable dissociation of storage and retrieval defects for nonverbal or pattern information was recorded during stimulation of homologous sites over the right cerebral cortex.

In separate investigative efforts, stimulation in the thalamus in the pulvinar nucleus produced comparable results. At one behavioral level, left, not right stimulation produced dysphasia, object-naming difficulties; right pulvinar stimulation caused the patients to commit pattern discrimination errors. With left pulvinar stimulation there was dysphasia and a sharp loss of immediate memory for verbal memoranda indicating that the patients may have experienced a brief retrograde memory loss. With right pulvinar stimulation, pattern discrimination and retrograde memory errors were also recorded. In each instance, however, the error level did not approach that seen during cortical stimulation.

The current findings reveal several significant functional differences between cortical and subcortical stimulation:

1. The intensity level of the stimulation burst, necessary to produce repeatable speech and language errors was higher for pulvinar sites than for the cortex. Moreover, even though the thalamus was stimulated more frequently (and with higher current) than the speech cortex, fewer errors resulted.
2. Different classes of anomie errors were observed. More substitution or misnaming errors were noted with left pulvinar stimulation, whereas stimulation of the primary cortical language area generally produced more omission errors.
3. A major dissociation between dysphasia and amnesia was noted. If the patients were unable to name an object during cortical stimulation, they also failed to recall the name of the object after stimulation. With pulvinar stimulation in contrast the patients frequently recalled the name of the object which they were unable to name or had incorrectly identified 6 to 12 seconds earlier. Finally, pulvinar aphasia was not always coupled with retrograde memory defects, the patient being unable to name an object in view, and yet being able to recall the name of the preceding object at the same time.

These data suggest that the pulvinar nucleus in the thalamus may interface the primary sensory cortex and the language brain, and sensory messages routed through the thalamus are attenuated or sharpened, and presented to the cortical stations for identification and storage.

Overall, the effects of cortical lesions in the language zone appear to be more severe and disabling than effects from similar lesions in subcortical thalamic zones. In man, recent and remote memory for materials and information may be served by a common retrieval mechanism situated within the cortical language zone, interacting with the subcortical regions.

Significance to Biomedical Research and the Program of the Institute: These investigations contribute to the basic understanding of the development and organization of structural-functional relations in the brain of man. This research advances clinical knowledge of the relationships between brain dysfunctions and amnesia, dysphasia, dyslexia and specific behavioral or adaptive responses.

Proposed Course of the Project: Tests are being designed to examine adaptive strategies used by neurologic patients to compensate for visuomotor or language disorders. Visual and auditory tasks will be developed to further delineate immediate and long-term memory impairment in patients with lateralized cortical and subcortical lesions.

Publications: Fedio, P. and Van Buren, J. Thalamo-cortical mediation of perception and memory in man. Proceedings of the XXVIII International Congress of Physiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02269-04 CN															
PERIOD COVERED October 1, 1979 through September 30, 1980																	
TITLE OF PROJECT (80 characters or less) Visual Evoked Potentials in Clinical Neurology and Neuro-Ophthalmology Former Title: Photic Flash Visual Evoked Potentials in Clinical Neurology and Neuro-Ophthalmology																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																	
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OTHER:	M. Seyal, M.D.	Clinical Associate	ET	NINCDS													
	S. Sato, M.D.	Acting Chief	CN	NINCDS													
COOPERATING UNITS (if any) Epilepsy Section, ETB, NINCDS																	
LAB/BRANCH Clinical Neurosciences																	
SECTION Clinical Neurophysiology																	
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205																	
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">TOTAL MANYEARS:</td> <td style="width: 33%;">PROFESSIONAL:</td> <td style="width: 34%;">OTHER:</td> </tr> <tr> <td style="text-align: center;">0.5</td> <td style="text-align: center;">0.2</td> <td style="text-align: center;">0.3</td> </tr> </table>			TOTAL MANYEARS:	PROFESSIONAL:	OTHER:	0.5	0.2	0.3									
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SUMMARY OF WORK (200 words or less - underline keywords) An analysis of the morphology, amplitude and latency of <u>visual evoked potentials</u> to photic flashes and reversing checkerboard pattern being conducted. Normative data so far have been collected from some 70 individuals, predominantly of 20-39 years. Visual evoked responses also have been examined in some 60 patients with various neurological disorders. Prolonged latencies of the major positive peak have been noted in patients with multiple sclerosis and other neurological disorders.																	

Project Description:Objectives:

The practicability and diagnostic value of this test has been and continues to be evaluated in Clinical Neurology. The examination of normal individuals and patients with various neurological disorders will aid our understanding of the mechanism of the VEP.

Methods Employed:

Initially, photic flash was predominantly used but soon it was found that results were inconsistent intra-as well as inter-individually. Therefore, the mode of stimulation was changed to reversing checkerboard pattern. However, flash stimulation has been used in patients who are uncooperative or unconscious. Evoked responses are averaged by the Nicolet CA 1000, analyzed on line and recorded with X-Y plotter. The routine electrode montage consists of O1, O2 and OZ referred to FZ.

Major Findings:

Normative data: Latencies of the major positive peak.

<u>Males</u>	<u>N</u>	<u>Right Eye stimulation</u>	<u>Left eye stimulation</u>
10-19 years	10	101.5 \pm 4.6 msec	100.5 \pm 4.0 msec
20-29 years	27	98.2 \pm 4.1 msec	98.5 \pm 3.9 msec
30-39 years	41	100.5 \pm 5.4 msec	100.2 \pm 5.5 msec
40-49 years	11	105.6 \pm 4.0 msec	106.4 \pm 5.5 msec
<u>Females</u>			
20-29 years	18	102.3 \pm 4.6 msec	101.6 \pm 3.5 msec

In MS patients, these latencies were 129.1 \pm 18.0 msec for right eye stimulation and 133.9 \pm 16.5 msec for left eye stimulation. Prolonged latencies were also noted in patients with amyotrophic lateral sclerosis, gyrate atrophy of the retina, proptosis, and hydrocephalus.

Significance to Biomedical Research and the Program of the Institute:

Evoked potentials are useful in the detection of occult lesions in the nervous system, and in establishing the presence of visual function in patients with extensive neurologic disease. The exclusive and varied patient population in NINCDS would provide an opportunity to study evoked potentials in a variety of disease entities.

Proposed Course of Projects:

This project will continue.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02431-01 CN								
PERIOD COVERED October 1, 1979 through September 30, 1980										
TITLE OF PROJECT (80 characters or less) Experimental Epilepsy: Seizures Produced by Kindling In Rat										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">S. Sato, M.D. Acting Chief</td> <td style="width: 10%;">CN</td> <td style="width: 30%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>M. Seyal, M. D. Clinical Associate</td> <td>ET</td> <td>NINCDS</td> </tr> </table> 			PI:	S. Sato, M.D. Acting Chief	CN	NINCDS	OTHER:	M. Seyal, M. D. Clinical Associate	ET	NINCDS
PI:	S. Sato, M.D. Acting Chief	CN	NINCDS							
OTHER:	M. Seyal, M. D. Clinical Associate	ET	NINCDS							
COOPERATING UNITS (if any) Epilepsy Section, ETB, NINCDS										
LAB/BRANCH Clinical Neurosciences										
SECTION Clinical Neurophysiology										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205										
TOTAL MANYEARS: <div style="text-align: center;">0.8</div>	PROFESSIONAL: <div style="text-align: center;">0.6</div>	OTHER: <div style="text-align: center;">0.2</div>								
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords) <u>Seizures produced by chronic stimulation (Kindling)</u> are a good model for <u>human epilepsy</u> . In rat, seizures are produced by daily electrical stimulation of <u>amygdaloid complex</u> and other central nervous system sites. In this project, Kindling of the various sites of the central nervous system, interictal project, Kindling of the various sites of the central nervous system, interictal epileptiform discharges and their propagation, and effect of sleep-wake cycles and maturation on the epileptiform discharges are being investigated.										

Project Description:

Objectives:

To increase the understanding of mechanism of epileptic seizures. Kindled seizures produced by daily electrical stimulation in rats are being studied from the neurophysiological point of view.

Methods Employed:

Male Sprague-Dawley rats weighing between 350 and 400 grams are anesthetized by intraperitoneal Pebtibarbutak abd a biopolar stainless steel electrode or tungsten electrode is stereotaxically implanted in the amygdala or other locations. Screw electrodes are also secured in the skull to monitor the cortical EEGs. The rats daily stimulation starts. The stimulation consists of bipolar pulses of 100 to 200 ma, 60 Hz and 1 second duration, and is given once a day. The EEG and behavioral manifestitations are observed before, during, and after stimulation. The rats are stimulated until spontaneous seizures are observed. Long term recording of the EEGs also are made periodically to observe spontaneous interictal discharges and their propagation. As the completion of the experiment, the rats are sacrificed and perfused with formaline and ferrocyanide for histological confirmation of the electrode position.

Major Findings:

None

Significance to Biomedical Research and Program of the Institute:

The Kindling model of epilepsy is unique in that chronic stimulation at the same intensity (initially subthreshold) eventually produces epileptic seizures. This model is analagous to chronic human epilepsy. Further understanding of kindled seizures in rats will in turn elucidation of the mechanism of the human epilepsy

Proposed Course of Project:

This project will continue.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER <p style="text-align: center; font-size: 1.2em;">Z01 NS 02432-01 CN</p>															
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>																	
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Brainstem Auditory Evoked Potentials in Clinical Neurology</p>																	
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SECTION <p style="text-align: center;">Clinical Neurophysiology</p>																	
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TOTAL MANYEARS: <p style="text-align: center;">0.5</p>	PROFESSIONAL: <p style="text-align: center;">0.2</p>	OTHER: <p style="text-align: center;">0.3</p>															
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<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS																
SUMMARY OF WORK (200 words or less - underline keywords) <p>Analysis of the morphology, amplitude and latency of <u>brainstem auditory evoked responses</u> to clicks is being conducted. Normative data have been collected from some 50 normal subjects, predominantly of 20-29 years. The test has been carried out in 14 patients with <u>various neurological disorders</u>. Prolonged latencies and distortion of morphology have been observed in patients with Multiple Sclerosis and Spinocerebellar degeneration.</p>																	

Project Description:

Objectives:

The practicality and diagnostic value of this test has been and continues to be evaluated in the Clinical Neurology. The examination in normal subjects and patients with various neurological disorders will help our understanding of the mechanism of brainstem auditory evoked potentials.

Methods Employed:

Clicks of 10-11Hz, 70dB above threshold generalized Nicolet 1007 and delivered to the testing ear through a head phone and masking noise is delivered to the contralateral ear at 40dB above the threshold. The brainstem auditory responses are recorded from CZ referred to A1 and A2, and averaged 1000 to 2000 times by the Nicolet CA 1000. The final record is made by a N-Y plotter.

Major Findings:

Some of the normative data are as follows:

MAJOR POSITIVE PEAKS (MSEC) - LEFT EAR

Males	I	II	III	IV	V
10-19 years	1.58+0.05	2.73+0.12	3.96+0.28	4.87+0.29	5.56+0.20
20-39 years	1.70+0.17	2.75+0.17	3.83+0.17	4.92+0.24	5.65+0.26

MAJOR POSITIVE PEAKS (MSEC) - RIGHT EAR

10-19 years	1.64+0.06	2.81+0.04	3.94+0.14	4.85+0.23	5.70+0.15
20-39 years	1.77+0.21	2.80+0.22	3.80+0.22	4.99+0.19	5.78+0.22

Females (left ear)

20-39 years	1.71+0.10	2.76+0.23	3.77+0.18	4.62+0.29	5.66+0.16
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Females (right ear)

20-29 years	1.73+0.16	2.87+0.19	3.82+0.12	4.71+0.11	5.66+0.11
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Prolongation of latencies was noted in patients with Multiple Sclerosis.

Significance to Biomedical Research and the Program of the Institute:

Evoked potentials are useful in the detection of occult lesions in the nervous system, particularly in the brainstem-auditory path-

way. The extensive and varied patient population in NINCDS would provide an opportunity to study evoked potentials in a variety of disease entities.

Proposed Course of Project:

This project will continue.

Publications:

None

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Developmental and Metabolic Neurology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT

October 1, 1979 through September 30, 1980
Developmental and Metabolic Neurology Branch
National Institute of Neurological and Communicative Disorders and Stroke
Roscoe O. Brady, Chief

The Branch continued its multilateral approach to the etiology and therapy of neurological diseases in FY 80. Notable progress has been made in several major aspects of these endeavors. The principal research efforts of the Branch concern the following objectives. 1. Basic and clinical investigations of the metabolism of complex lipids and mucopolysaccharides in normal and pathologic states. 2. The organization and function. 3. Elucidation of the role of myelin glycoproteins in the development and maturation of the nervous system and their role in the pathogenesis of demyelinating diseases. 4. The function of enzymes on the external surface of cells. The important accomplishments in each of these areas in the past year are summarized in this report.

I. INHERITED METABOLIC DISEASES

A. Enzyme Replacement in Lipid Storage Diseases

One of the major contributions of the Branch this year was the demonstration that several patients with Gaucher's disease in our clinical protocol appear to be improved as a consequence of enzyme replacement. Two young males with this disorder were maintained on a prospective regimen consisting of intravenous infusion of the deficient enzyme every other month. It is our impression that the boys are much improved as a result of this intervention. This feeling is shared by the local pediatricians who provide the general care for these patients. During the past two and one-half years the recipients have not required splenectomy that was imminent because of severe thrombocytopenia in the younger boy and was anticipated for the older child. The platelets are now in the normal range in both patients. There has been no untoward reaction to the exogenous enzyme, and the recipients have not become sensitized to it. Although enzyme replacement did not appear to cause a detectable reduction in the size of the enlarged liver and spleen, further progression of the organomegaly was halted by enzyme replacement.

Another potentially important observation concerning enzyme replacement occurred in a female patient with Gaucher's disease who was treated with corticosteroid prior to infusion of the enzyme. She was entered on our protocol at the insistence of an orthopedic surgeon who had performed two hip replacements and a prosthetic operation on one of her knees because of the bone involvement that can occur in this disorder. It is the surgeon's impression that the remaining knee joint is improved. While we require more definitive data in this regard, it may be noted that the patient has not required a prosthetic procedure on the second knee joint now some ten months following enzyme infusion.

B. Specific Targeting of Exogenous Enzymes

Although we are greatly encouraged by these results, we believe that we can improve further on the delivery of enzymes to the storage cells in patients with heritable metabolic disorders. We have increased the delivery of glucocerebrosidase (the enzyme lacking in patients with Gaucher's disease) to nonparenchymal cells by 45-fold in the livers of rats by selectively exposing N-acetylglucosamine and mannose residues on the oligosaccharide portion of the molecule. These modifications markedly enhanced absorptive pinocytosis by these cells. Since nonparenchymal macrophages in liver and other organs are the principal sites of glucocerebrosidase storage in Gaucher patients, we anticipate that the clinical use of enzyme modified in this manner will result in enhancement of its therapeutic efficacy.

C. Delivery of Enzymes to the Central Nervous System

Our studies on the modification of the blood-brain barrier in primates has progressed in a most satisfactory fashion. The nervous system of monkeys whose barriers have been altered on a number of occasions has been subjected to complete histological analyses. Our observations indicate that this procedure did not provoke pathologic changes in the brain or spinal cord. However, a temporary effect on the pigmented epithelium of the eye was noted. These lesions appeared to heal in time without permanent damage.

We have extended our investigations of enzyme delivery to the central nervous system in a collaborative undertaking with Dr. Edward Neuwelt, a neurosurgeon at Southwestern Medical School in Dallas. Using ^{125}I -labeled hexosaminidase A (the enzyme lacking in patients with Tay-Sachs disease), We have discovered that barrier permeability exists for only a very brief time for this macromolecule. We plan to determine the optimal period for the administration of this enzyme in rodents and in dogs. We anticipate that human trials will ultimately follow. Dr. Neuwelt has already performed barrier modification in humans with metastatic lesions in the central nervous system on a number of occasions. He has not observed untoward effects from this procedure, and contrary to our observation in monkeys, he found no indication of eye pathology in his investigations with humans. If these studies continue to progress satisfactorily, it is expected that enzyme replacement for human metabolic disorders will be attempted in the relatively near future.

II. MEMBRANE RECEPTORS FOR ENVIRONMENTAL SIGNALS

Further work on the role of gangliosides as biotransducers of membrane-mediated information has centered on fundamental studies concerning the mechanism of action of these surface components. A portion of this research involved chemical modification of gangliosides to determine which particular components of the molecule are required for interaction with the external ligand and its coupling with adenylate cyclase. Evidence has been obtained for the presence of a novel translocating protein in cell membranes that functionally links receptors for the external ligand to the adenylate cyclase

complex. Related work has utilized cross-linking reagents to identify components of trophic hormone receptors. A potentially important result of a companion study with beta-adrenergic receptors was the discovery that the responsiveness of cultured cells to catecholamines diminishes with time in culture. This effect may be similar to changes that occur with aging in humans and its use as a model of senility will be explored.

III. MULTIPLE SCLEROSIS

Work has continued on the purification and characterization of the major myelin-associated glycoprotein (MAG) discovered by this laboratory. Three major advances have been made in this area in the past year. The first was the development of a highly sensitive radioimmunoassay that permits for the first time the measurement of MAG in tissue samples without prior purification of myelin and extraction of the glycoprotein. The quantity of MAG in the central and peripheral nervous systems has been determined as well as in myelinating tissue cultures. We propose to use this technology to quantitate the dramatic qualitative changes in MAG that we reported last year in tissues from patients with multiple sclerosis. The second development was the demonstration that purified human myelin contains a neutral protease that readily cleaves MAG to a smaller polypeptide. This enzyme is activated by ammonium bicarbonate and other salts. Human MAG appears to be much more susceptible to this enzyme than MAG in lower animals. We propose to examine the detailed kinetics of this reaction and to determine whether this alteration of MAG is involved in the pathogenesis of demyelinating diseases. The third major discovery was the demonstration (with LNNS) that the quantity of MAG that can be detected by immunocytochemical analysis is diminished before myelin basic protein is altered in the brains of patients with progressive multifocal leukoencephalopathy, a disease caused by viral infection of oligodendrocytes. This observation is completely consonant with the rapid loss of MAG in developing multiple sclerosis plaques we reported previously. These observations, combined with the current extensive interest in neutral proteases in demyelinating diseases, strongly implicate alterations in MAG in the pathogenesis of these disorders.

IV. ECTO-ENZYMES

Emphasis has continued on an examination of the characteristics and roles of plasma membrane enzymes (ectoenzymes) that react with external substrates. One of our approaches to this study has been the use of covalent binding probes that exhibit enhanced specificity for the particular enzyme in question. These probes have been employed to label ectoenzymes in situ. It was concluded that ectoenzymes are comprised of: (i) a protein moiety; (ii) a glycoprotein residue; and (iii) a phospholipid phase. The ectoenzymes under investigation in our Branch are probably involved in modulating membrane permeability and the threshold of excitability of various cells in the nervous system. Most, if not all of these cells exfoliate microvesicles that are enriched in ectoenzyme activities. Our experiments indicate that these vesicles (exosomes) serve a transport function between cells. This discovery is particularly significant since neuronal cells are supplied with a variety of proteins from glial cell matrices and these proteins appear to be transferred in discrete quanta. The physiological contributions of the uniquely localized ectoenzymes are under comprehensive examination.

V. CONCLUDING STATEMENT

Of necessity, a summary such as this represents a highly selected overview of experiments underway in the Branch. It seems likely that many of the other findings that are described in the individual project reports will in time acquire a significance and degree of importance that is not immediately perceived. For example, in a series of studies related to our investigation of Gaucher's disease, we discovered a novel mammalian enzyme with extraordinary species and tissue specificity that catalyzes the hydrolysis of the terminal molecule of glucose of amygdalin (Laetrile). The two molecules of glucose in this compound must be cleaved before its potential tumoricidal activity can occur through the release of cyanide. The implications of these findings for cancer chemotherapeutic trials with this agent are weighty. At a minimum, it would seem to be necessary to demonstrate increased activity of these glucosidases in the tumor cells to be treated with this compound over that in normal cells before its use can be considered appropriate.

CONTRACT NARRATIVE

Developmental and Metabolic Neurology Branch

Intramural Research Program, NINCDS

October 1, 1979 through September 30, 1980

Contractor: NEW ENGLAND ENZYME CENTER, TUFTS UNIVERSITY(N01-NS-5-2321)

Title: Preparation of Glucocerebrosidase from Human Placental Tissue

Contractor's Project Director: Henry E. Blair

Current Annual Level of Support: \$200,000

Objectives: To isolate human placental glucocerebrosidase in sufficient purity and quantity so that it can be used in enzyme replacement trials in patients with Gaucher's disease.

Major Findings: A procedure has been developed for the large-scale purification of human placental glucocerebrosidase that is of sufficient purity and catalytic activity that it can be safely administered to humans with Gaucher's disease. The intravenous infusion of this enzyme to two young patients with this disorder has brought about the following effects: (1) the progressive enlargement of the spleen and liver of these patients has been arrested. (2) Their diminished blood platelet count has been restored to the normal range. (3) The general health and vigor of the recipients has been dramatically improved.

Significance to Biomedical Research and to the Program of the Institute: One of the principal missions of the Institute is to develop effective therapies for the treatment of human diseases. If the results obtained in the initial trials of prospective enzyme replacement therapy discussed in the preceding paragraph can be confirmed, we will have accomplished an unprecedented medical feat.

Proposed Course of the Contract: We plan to expand the number of recipients of enzyme replacement in Gaucher's disease to determine whether the initial salutary findings can be substantiated. We shall also seek to chemically modify the enzyme so that it is more efficiently delivered to the specific cells that store the accumulating lipid. Finally, we shall investigate the possibility of altering the blood-brain barrier to try to deliver the enzyme to the central nervous system in patients with the neuronopathic form of this disease.

CONTRACT NARRATIVE

Developmental and Metabolic Neurology Branch
Intramural Research Program, NINCDS
October 1, 1979 through September 30, 1980

Contractor: WEIZMANN INSTITUTE OF SCIENCE (N01-NS-5-2300)

Title: Production of Three Radiolabeled Glycolipid Substances

Contractor's Project Director: David Shapiro, Ph.D.

Current Annual Level of Support: \$54,000

Objectives: The enzymatic defects in heritable sphingolipid storage disorders in humans is ultimately best diagnosed through the use of radioactively labeled natural lipid substrates. The Weizmann Institute of Science provides the NIH with radioactive carbon-¹⁴ labeled glucocerebroside, sphingomyelin, and ceramidetrihexoside for the diagnosis of patients and detection of carriers of Gaucher's disease, Niemann-Pick disease, and Fabry's disease respectively.

Major Findings: The principal investigator is a world-recognized expert in the chemical synthesis of sphingolipids. He has devised procedures for incorporating radioactive carbon-¹⁴ into critical portions of sphingolipid molecules. Using these substrates, we incubate human tissue specimens to determine the activity of glucocerebrosidase sphingomyelinase and ceramidetrihexosidase enzymes. These determinations permit us to diagnose patients with the disorders listed above, to identify heterozygous carriers of these metabolic diseases, and to monitor pregnancies at risk for any of these conditions. These labeled lipids are also required to monitor purification procedures for the preparation of enzymes for therapeutic replacement trials.

Significance to Biomedical Research and to the Program of the Institute: The ability to diagnose patients, identify heterozygotes, and monitor pregnancies at risk for any of the known lipid storage diseases represents major contributions to the control of the incidence of the sphingolipidoses at the present time.

Proposed Course of the Contract: The contractor will provide necessary radioactive sphingolipids for diagnostic tests. He will also make these compounds for monitoring enzyme purification procedures. He will develop sphingolipid analogues for the production of genetic animal models of human lipid storage diseases.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 00706-21 DMN																												
PERIOD COVERED October 1, 1979 through September 30, 1980																														
TITLE OF PROJECT (80 characters or less) Inborn Errors of Metabolism of Diverse Etiology.																														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 60%;">John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section</td> <td style="width: 10%;">DMN</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td>Other:</td> <td>George Constantopoulos, Ph.D. Staff Biochemist</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Daniel W. Stowens, M.D. Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Edward I. Ginns, M.D., Ph.D. Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Jan K. Steusing Research Assistant</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Nancy Krett Guest Worker</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Roscoe O. Brady, M.D. Chief</td> <td>DMN</td> <td>NINCDS</td> </tr> </table>			PI:	John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section	DMN	NINCDS	Other:	George Constantopoulos, Ph.D. Staff Biochemist	DMN	NINCDS		Daniel W. Stowens, M.D. Clinical Associate	DMN	NINCDS		Edward I. Ginns, M.D., Ph.D. Clinical Associate	DMN	NINCDS		Jan K. Steusing Research Assistant	DMN	NINCDS		Nancy Krett Guest Worker	DMN	NINCDS		Roscoe O. Brady, M.D. Chief	DMN	NINCDS
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SUMMARY OF WORK (200 words or less - underline keywords) A better understanding of <u>metabolic disorders</u> which affect the nervous system is the goal of this project. In some phases, the studies are purely <u>diagnostic</u> and are applied to assist in diagnosing the <u>less common disorders of metabolism</u> . Other phases deal with biochemical observations in known disorders that suggest steps in the <u>pathogenesis</u> of the disease. In some poorly understood groups of <u>neurologic disease</u> , studies are conducted to draw biochemical correlations where none had previously been known or were poorly developed. <u>Therapeutic trials</u> are conducted in selected disorders.																														

Project Description:

Objectives: The majority of chronic neurological disorders are first recognized during childhood. Because of the chronicity and long duration of the handicaps these diseases constitute a formidable medical and social problem. Taken together, mental retardation (frequently familial), birth defects, cerebral degenerations, and inborn errors of metabolism affecting the nervous system amount to over four million people in the U.S.A. Our main objectives are: 1) to study the pathogenesis and etiology of these diverse disorders which are frequently of genetic origin, 2) to devise special diagnostic tests including identification of heterozygotes, 3) to institute therapeutic modifications of the respective disorders, 4) explore preventive measures and prenatal diagnosis.

Patient Material: Patients with the following disease categories were admitted for investigation; sphingolipidoses, mucopolysaccharidoses, ceroid lipofuscinosis, spinocerebellar degeneration, congenital pyruvic and lactic acidosis, glycogen storage disorders, and adrenoleukodystrophy. A number of patients with unknown diseases were admitted for study.

Methods Employed:

- 1) Neurologic, developmental and genetic assessments of the patients were made, including family studies when appropriate.
- 2) Determination of profiles of lipids, amino acids, proteins, mucopolysaccharides and carbohydrates in various tissues and, when appropriate, in urine and cerebrospinal fluid.
- 3) Assay of enzyme activities in the peripheral blood leukocytes and platelets of genetic diseases studied.
- 4) Establishment of skin fibroblast tissue cultures in patients with genetic disorders for study of enzyme activity and turnover studies using radioactive substances.
- 5) Employment of invasive techniques, if required, for definitive diagnosis. Brain and liver biopsies are performed and the tissues are used for biochemical, chemical, enzymatic and electron microscopic studies.
- 6) Therapeutic modification of the diseases is attempted whenever possible. For this purpose, pharmaceuticals, hormones, plasma or formed blood elements transfusion, dietary modifications and, where appropriate, enzyme replacement are used.
- 7) In case the patient with a metabolic disease dies, samples of the organs and other tissues are stored frozen for future chemical and enzymatic studies; the fresh specimens of tissue are immediately fixed or processed for histochemical and electron microscopic studies.

Major Findings:

1. A portion of our efforts were directed toward an understanding of the pathogenesis of the mucopolysaccharidoses. The most important contributions have been in two areas, a) determination of the content and composition of mucopolysaccharides in the body fluids and tissues of patients with different types of this disorder; b) the demonstration of glycolipid abnormalities in those types of mucopolysaccharidoses that have mental retardation as a complication. Parallel findings have been made in isolated glial and neuronal fractions.
2. Concentrated efforts were made to introduce therapeutic modifications of selected inborn errors of metabolism. In an attempt to delineate the pathogenesis and clinical variability of the sphingolipidoses, and in order to proceed logically with therapeutic modalities, principally enzyme replacement, these disorders have been studied in depth. Gaucher's disease has been particularly closely scrutinized. Suggestions from the literature and observations of our patients have prompted us to investigate the significance of disturbances of liver function, lung function, immune response, cardiac function, and reticuloendothelial function. Results of some of these studies are cited in appropriate listed publications.
3. Patients presenting with myoclonus are being investigated. Four patients with the diagnosis of Lafora body disease have been identified. Clinicopathologic correlation has been made. The diagnostic value of the liver pathology has been confirmed. The nature of the biochemical defect is being investigated. Preliminary characterization of the storage material in liver and identification of a previously unknown urinary polysaccharide have been accomplished.
4. Patients with ataxia are being investigated for biochemical disorders. Two patients with ataxia have been demonstrated to have "ragged red fibers" in their muscle mitochondria. These patients have lactic and pyruvic acidemia and thus likely have some error of oxidative metabolism. No deficiency of the pyruvate dehydrogenase complex has been detected in these or other similar patients. The precise biochemical lesion is being pursued. Other causes of hereditary familial ataxia currently being actively tested are pyruvate dehydrogenase deficiency, hexosaminidase variants, and other variants of the sphingolipidoses. No patients with Friedreich's ataxia or olivopontocerebellar atrophy have been found to be deficient in any of these enzymes.
5. Modification of the blood-brain barrier results in the entry of macromolecules such as enzymes into brain interstitial fluid. We have further demonstrated that catalytically active enzymes are taken up and incorporated into lysosomes of neurons and to some extent glia. The procedure can be carried out safely and can be monitored noninvasively. The possibility of enzyme replacement in

the central nervous system is being investigated. Furthermore, the receptors on neurons for macromolecules are being described. Studies designed to describe the processing of macromolecules by brain are being carried out.

6. Pilot studies in Fabry's disease indicate that the unmetabolizable lipid, ceramide trihexoside, can be removed by plasmapheresis. The kinetics of reappearance in the serum suggests that an exchangeable pool of the lipid exists. Tissue concentrations of the lipid and clinical correlation will be made after multiple exchanges over a period of six months to one year.

Significance to Biomedical Research and the Program of the Institute:

Because the majority of infections affecting man are now under quite satisfactory control, the time has come for increased attention to accord a measure of control to such common disorders as hereditary diseases, congenital malformations, mental retardation, and degenerative conditions affecting the nervous system. Improved methodology makes it now feasible to advance our knowledge and institute some control in certain of these crippling chronic disorders. Prevention and therapy include prenatal diagnosis, enzyme infusion, dietary modifications and institution of certain eugenic measures. Since many of the disorders affect exclusively or predominantly the nervous system, the study of etiology and pathogenesis as well as institution of therapeutic trials are of importance in furthering the main mission of our Institute.

Proposed Course of the Project: During the next years increasing emphasis will be given to the pathogenesis of the hereditary diseases and to therapeutic modifications of respective disorders. We expect to evaluate the usefulness of enzymes in the treatment of various diseases. Further, the possibility of plasmapheresis as a therapeutic tool in storage disorders will be more thoroughly investigated.

Publications:

1. Askanas, V., Engel, W. K., Britton, D. E., Adornato, B. T. and Eiben, R. M.: Reincarnation in cultured muscle of mitochondrial abnormalities. Arch. Neurol., 35: 801-809, 1978.
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 00815 20 DMN																		
PERIOD COVERED October 1, 1979 through September 30, 1980																				
TITLE OF PROJECT (80 characters or less) Metabolism of Complex Lipids of Nervous Tissue																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
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COOPERATING UNITS (if any) Weizmann Institute of Science, Rehovot, Israel Tufts University Medical School, Boston, Massachusetts National Center for Toxicological Research, Jefferson, Arkansas																				
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SUMMARY OF WORK (200 words or less - underline keywords)																				
<p>Procedures are developed for the purification of enzymes from tissues such as human placenta that are lacking in patients with <u>Gaucher's disease</u>, <u>Fabry's disease</u>, <u>Tay-Sachs disease</u> and <u>Niemann-Pick disease</u> and <u>mannosidosis</u>. The effects of <u>enzyme replacement therapy</u> in patients with these disorders is under investigation. Procedures are developed for the diagnosis of patients with these disorders, the <u>detection of heterozygous carriers</u> of these genetic traits, and for the <u>monitoring of pregnancies at risk</u> for each of these diseases.</p>																				

Project Description:

Objectives: (1) To elucidate the biosynthetic pathways for the formation of long chain fatty acids, cerebroside, gangliosides and sphingomyelin; (2) to study the control mechanisms which regulate these processes; (3) to study the metabolic fate of sphingolipids in normal and lipodystrophic disease states, and (4) to provide diagnostic and therapeutic procedures for the amelioration and control of the lipid storage diseases.

Methods: Glucocerebroside and galactocerebroside labeled with ^{14}C in either the hexose or fatty acid portion of the molecule have been synthesized. ^{14}C -labeled sphingomyelin and gluco- and galactopycosine have been similarly prepared. Ceramide-trihexoside and ceramide tetra hexoside (globoside) specifically labeled with radioactive hydrogen- ^3H have been prepared. The metabolism of these labeled materials has been investigated in vivo and in vitro. Human placenta has proved to be a convenient and rich source of sphingolipid hydrolases. Isolation of these enzymes for therapeutic replacement trials is a major continuing portion of this project.

Major Findings: (1) Enzyme replacement in Gaucher's disease and Fabry's disease holds promise as an effective therapeutic procedure for the amelioration of these disorders. A long lasting reduction of blood glucocerebroside, the accumulating lipid in Gaucher's disease was obtained in patients infused with purified human placental glucocerebrosidase. Patients with Gaucher's disease have subnormal activity of this enzyme in their tissues. We have developed a method for the purification of glucocerebrosidase on a large scale in a form that is suitable for administration to humans. Enzyme replacement trials in Gaucher's disease are underway with this preparation. The clinical course of the disease was greatly improved in two young boys who received the enzyme. Accordingly, they are being followed on a prospective long-term course of enzyme replacement. Other trials resulted in dramatic reductions in the quantity of accumulated glucocerebroside in the liver of two adult patients who received a short course of corticosteroid prior to administration of the enzyme. Furthermore, pathologic bone changes in the knee of a Gaucher patient appears to have been improved by her therapeutic combination.

(2) We have developed a method for the purification of sphingomyelinase, the enzyme lacking in Niemann-Pick disease, also from human placental tissue. At the present time, it is very difficult to obtain sufficient quantities of this enzyme for replacement therapy trials. However, in collaboration with the National Center for Toxicological Research, we have discovered a strain of Balb/c mice with an autosomal recessive

neurological degenerative disorder characterized by the accumulation of several sphingolipids. These animals exhibit persistent deficiency of sphingomyelinase and glucocerebrosidase that is evident immediately after birth. We propose to utilize this important animal model for studies of enzyme replacement therapy.

(3) We continue to serve as a center for the diagnosis of patients and detection of carriers for all of the lipid storage diseases and much of our effort is devoted to the monitoring of pregnancies at risk for heritable metabolic disorders. During the past year, we performed more than 220 diagnostic assays for physicians and genetic counselors from all over the world.

Significance: Enzyme replacement appears promising for the treatment of Gaucher's disease and Fabry's disease. It is expected that the deleterious clinical course in these patients will be ameliorated by this form of therapy. The ability to introduce enzymes into the central nervous system has profound implications for the treatment of genetic disorders that cause brain damage.

Proposed Course: We will continue to carry out and monitor the long-term effects of enzyme infusion in patients with Gaucher's disease, Fabry's disease and other disorders. Studies of enzyme replacement with purified sphingomyelinase will be carried out in animal models of Niemann-Pick disease. We shall attempt to design systems to deliver these exogenous enzymes in a clinically useful fashion.

Publications:

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Biochem. Biophys. Res. Commun. 94: 199-204, 1980.
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01309-15-DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Biosynthesis and Function of Glycosphingolipids and Other Glycoconjugates		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div style="width: 70%;"> PI: P. H. Fishman, Ph.D., Research Biochemist OTHER: R. Rebois, Ph.D., NIH Fellow R. O. Brady, M.D., Branch Chief </div> <div style="width: 25%; text-align: right;"> DMN, NINCDS DMN, NINCDS DMN, NINCDS </div> </div>		
COOPERATING UNITS (if any) Laboratory of Molecular Biology, NINCDS Laboratory of Cellular Metabolism, NHLBI		
LAB/BRANCH Developmental & Metabolic Neurology Branch		
SECTION Enzymology and Genetics		
INSTITUTE AND LOCATION NIH, NINCDS, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">1.9</div>	PROFESSIONAL: <div style="text-align: center;">1.4</div>	OTHER: <div style="text-align: center;">0.5</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) 1. <u>G_{M1}</u> was modified by removal of its fatty acid. The modified ganglioside was readily taken up by <u>G_{M1}</u> -deficient cells as measured by increased <u>cholera toxin</u> binding or by uptake of a tritium-labeled derivative. Cells treated with this derivative accumulated more <u>cyclic AMP</u> when exposed to toxin than did <u>G_{M1}</u> -treated cells even though equivalent amounts of toxin were bound to the cells. In addition, there was more activation of adenylate cyclase. Thus, the sphingosine moiety of <u>G_{M1}</u> appears to modulate toxin action. 3. Rat <u>testes</u> membranes were incubated with iodinated human <u>chorionic gonadotropin (hCG)</u> and then treated with <u>cross-linking reagents</u> . Covalently coupled hCG-membrane complexes were detected by inability of high salt or acid to displace the hormone. The detergent-solubilized cross-linked complex behaved on gel filtration columns and sucrose density gradients similar to the uncross-linked <u>hormone-receptor complex</u> . By SDS-polyacrylamide gel electrophoresis, the crosslinked complex had a smaller molecular size than by the above methods, thus, indicating that the <u>receptor</u> may be a <u>multimeric</u> complex.		

Project Description:

Objectives: To investigate the function of membrane glycosphingolipids in the regulation of cell proliferation, cell morphology, hormone action and toxin sensitivity; to explore the regulation of glycosphingolipid findings to anabolic heritable disorders; to determine the underlying mechanism of altered glycosphingolipid biosynthesis in neoplastic tissues; these studies are being extended to other membrane glycoconjugates.

Methods: Gangliosides radiolabeled in selected portions of the molecule are prepared by specific enzymatic reactions. Thus, GM₁ labeled with tritium in the terminal galactose is prepared by oxidation with galactose oxidase and then reduction with sodium borotritide. GM₁ is deacylated by refluxing with barium hydroxide in butanol, N-acetylated with acetic anhydride in pyridine and purified by silica gel column chromatography. Purity and composition of the modified product is assessed by thin-layer and gas-liquid chromatography.

GM₁-deficient transformed mouse fibroblasts (NCTC-2071 cells) and rat glioma C6 cells are continuous cell lines and are grown in monolayer cultures. The cells are incubated with GM₁ or modified GM₁ in serum-free medium, washed extensively and then analyzed for ganglioside uptake. This is determined by either using ³H-labeled gangliosides or by measuring the amount of [¹²⁵I]-cholera toxin that binds to the cells. Gangliosides are also extracted from the cells, purified, separated by thin-layer chromatography and detected by radioscanning the chromatograms.

Binding of cholera toxin and hormones to cells, cell membranes and liposomes is determined with ¹²⁵I-labeled proteins and either filtration or centrifugation techniques. Levels of cyclic AMP and adenylate cyclase activity are measured with a modified cyclic AMP protein binding assay. Gonadotropin binding particles are prepared from rat testes by differential centrifugation. Isolated Leydig cells are prepared from rat testes by treatment with collagenase and then differential centrifugation. Gonadotropin-receptor complexes are solubilized with detergents such as Triton X-100 and analyzed by gel filtration column chromatography, sucrose density gradient centrifugation and SDS-polyacrylamide gel electrophoresis.

Major Findings:A. Effect of Ceramide Moiety of GM₁ on Cholera Toxin Action

In previous studies, we demonstrated that GM₁ is the receptor for cholera toxin by its ability to sensitize GM₁-deficient cells to the toxin. As other homologous glycosphingolipids are ineffective and the toxin can bind to the free oligosaccharide of GM₁, it is apparent that the specificity for toxin binding resides in the oligosaccharide part

of the ganglioside. The lipid (ceramide) part of the molecule anchors it to the cell membrane but whether the lipid moiety plays any additional role in toxin action has not been investigated. We prepared a GM₁ derivative with the long chain fatty acid replaced by an acetyl group (DA-GM₁). When added to the culture medium, DA-GM₁ was taken up by GM₁-deficient NCTC-2071 and rat glioma C6 cells. Uptake increased with time of incubation and was proportional to the concentration of the ganglioside in the medium. Cells treated with DA-GM₁ bound more iodotoxin in proportion to the amount of DA-GM₁ incorporated by the cells. By using tritium-labeled DA-GM₁, we were able to show that most of the cell-associated radioactivity remained as DA-GM₁. When exposed to cholera toxin, DA-GM₁ treated cells accumulated more cyclic AMP than did control cells as had been shown previously for GM₁-treated cells. However, when GM₁-treated and DA-GM₁-treated cells bound equivalent amounts of toxin, the latter cells accumulated more cyclic AMP than did the former cells. In addition, adenylate cyclase was activated more in the latter than in the former cells. Additional experiments indicated that toxin affinity and the ratio of toxin bound to ganglioside taken up was similar for the two types of treated cells. The lag period was also the same and other N-acetylated glycosylsphingosines did not cause this effect. We propose that a toxin molecule bound to DA-GM₁ is more likely to activate adenylate cyclase than one bound to GM₁ and thus the ceramide moiety of the toxin receptor is important in modulating toxin action.

B. Chemical Cross-Linking of Gonadotropin to its Membrane Receptor

Rat testes contain a small number of high affinity binding sites for human chorionic gonadotrophin (hCG). These receptors are associated with the Leydig cells, which accumulate cyclic AMP and synthesis testosterone when exposed to hCG. The nature of the hCG has not yet been determined but previous studies have indicated that it is a large molecular weight protein (or glycoprotein). In addition, gangliosides and phospholipids may play a role in hormone binding and action. One approach to exploring the nature of hormone receptors and their association with other membrane components is to use chemical cross-linking reagents. We incubated testes membranes with [¹²⁵I]-hCG followed by addition of dithiobis(succinimidyl propionate) (DTSP), a homobifunctional reagent that reacts with amino groups under physiological conditions. The membranes were then washed and exposed to either 4 M MgCl₂ or pH 3, conditions which normally dissociate the hormone-receptor complex. After treatment with the cross-linking reagent, up to 50% of the bound hCG remained attached to the membranes. There was little or no cross-linking of labeled hCG to membranes previously exposed to an excess of unlabeled hormone.

Membranes incubated with [¹²⁵I]-hCG were treated with and without DTSP, washed and solubilized with Triton X-100. The solubilized hCG-receptor complexes were fractionated on gel filtration columns and on sucrose density gradients. The native and the cross-linked complexes

appeared to have the same molecular properties by these two techniques. The cross-linked complexes also were analyzed by SDS-polyacrylamide gel electrophoresis. The complex appeared to have an apparent molecular weight smaller than the size obtained by gel filtration and sucrose density gradient methods. Since glycoproteins migrate atypically on SDS-polyacrylamide gels, additional studies will be required to establish the exact molecular weight of the receptor. Our preliminary studies, however, indicate that the hCG receptor may be composed of more than one polypeptide, possibly a dimer of two binding subunits or a binding subunit associate with additional subunits. These latter subunits may provide specificity or regulatory functions.

We also have been able to immunoprecipitate the solubilized cross-linked hCG-receptor complex with antibodies against hCG. The antibody-hCG-receptor complexes were then absorbed to Protein A-agarose beads. This should provide a powerful and useful technique to purify the receptor as well as other membrane components that are associated with it. More recently, the anti-hCG antibodies have been directly coupled to polyacrylamide beads which appear to be effective in absorbing the solubilized cross-linked hCG-receptor complex.

Significance: These studies are providing information on the organization of membrane components and their function as receptors. Both the carbohydrate and lipid parts of ganglioside GM1 are important for its ability to act as a receptor for cholera toxin. The ability to covalently attach gonadotropin to its membrane receptor is a potent procedure for elucidating the structure and organization of this receptor.

Proposed Course: The project will be continued with emphasis placed on the purification and identification of the cross-linked hCG-receptor complex. The same techniques will be applied to the thyrotropin receptor in thyroid cells.

Publications:

1. Achtman, M., Jarett, L., Brady, R. O., Collier, R. J., Cuatrecasas, P., Dales, S., Helencus, A., Olsnes, S., Rosenbusch, J. P. and Tomasz, A. Penetration of exogenous macromolecules into cytoplasmic matrices. Life Sci. Res. Reports 11: 133-141, 1978.
2. Fishman, P. H. and Atikkan, E. A.: Induction of cholera toxin receptors in cultured cells by butyric acid. J. Biol. Chem. 254: 4342-4344, 1979.
3. Fishman, P. H. and Henneberry, R. C.: Induction of ganglioside biosynthesis in cultured cells by butyric acid. In Sweeley, C. C. (Ed.): Biochemistry of Cell Surface Glycolipids. ACS Symposium Series Vol. 128. Washington, D. C., American Chemical Society, 1980, 223-240.

4. Fishman, P. H., Moss, J., Richards, R. L., Brady, R. O. and Alving, C. R.: Liposomes as model membranes for ligand-receptor interactions: studies with cholera toxin and glycolipids. Biochemistry 18: 2562-2567, 1979.
5. Fishman, P. H., Pacuszka, T., Hom, B. and Moss, J.: Modification of ganglioside GM₁: effect of lipid moiety on cholera toxin action. J. Biol. Chem. 255: in press.
6. Macher, B. A., Pacuszka, T., Mullin, B. R., Sweeley, C. C., Brady, R. O. and Fishman, P. H.: Isolation and identification of a fucose-containing ganglioside from bovine thyroid gland. Biochim. Biophys. Acta 588: 35-43, 1979.

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Chemical Synthesis of Radioactive Sphingolipids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. E. Gal, Head, Neurochemical Methodology Section
OTHER: F. J. Fash, Bio. Lab. TechnicianDMN, NINCDS
DMN, NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Developmental and Metabolic Neurology Branch

SECTION

Neurochemical Methodology Section

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS☐ (b) HUMAN TISSUES☒ (c) NEITHER☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sphingolipids containing radioactive isotopes were synthesized and used for metabolic studies and as diagnostic tools in sphingolipidoses. ¹⁴C and ³H Labels were introduced by synthetic and semi-synthetic techniques, gas exposure, and a new approach: functional group exchange.

Project Description:

Objectives: To prepare sphingolipids labeled with radioactive isotopes. The compounds are used for metabolic studies and as diagnostic tools in investigations related to hereditary lipid storage diseases.

Methods and Major Findings: A multitude of approaches were used in labelling glycolipids such as chemical synthesis, partial synthesis, minor synthetic modifications, functional group exchange and tritium gas exposure. These methods could be classified into two categories: specific and non-specific labelling. The ideal approach is the specific labelling which consists of the tagging of a complex molecule at a pre-determined atom. Total synthesis is the best way to accomplish this but up to now only few sphingolipids have been synthesized. We synthesized sphingosine, psychosine and galactocerebroside specifically labelled by total synthesis. However, our main effort is directed toward methods which would allow specific labelling of atoms yet would not necessitate tedious syntheses. A promising technique which we developed is called the functional group exchange. A chemical group such as an acetyl or carboxyl is split from a molecule and is replaced with a similar but radioactive one. With this approach we could prepare aminosugars even gangliosides. Using the approach minor synthetic modification we prepared asialo ganglioside, Tay-Sachs ganglioside and ceramidetrihexoside. In this approach oxidation and reduction of an alcohol group in the molecule with a radioactive reducing agent would reestablish the original lipid in radioactive form. The lipids used as starting material for this approach were isolated from human tissues. Tritium gas exposure, a non-specific approach, was repeatedly used for labelling ceramide dihexoside, dihexoside and globoside. By this method all the non-labile hydrogen atoms in a molecule become radioactive. This procedure is relatively simple but the purification of the resulting compounds is complex. Also this type of compound requires more elaborate extractions for enzyme assays.

L-glucosylceramide was synthesized. This compound is a stereo-isomeric analogue of D-glucosylceramide that occurs in nature and accumulates in pathological quantity in the organs and tissues of patients with Gaucher's disease. The properties of L-glucosylceramide that have been examined so far have been found to be indistinguishable from the naturally occurring glycolipid. However, L-glucosylceramide is completely refractory to enzymatic hydrolysis by purified placental glucocerebrosidase and enzyme(s) present in whole tissue extracts.

Significance: The compounds are indispensable for the detection, identification and isolation of enzymes connected to lipid storage diseases. Also studies related to qualitative and quantitative determination of enzymes in animal or human tissues necessitate these labelled substrates. Prenatal diagnoses are of rising importance.

These labelled compounds play a key role in these diagnostic procedures. As a therapeutic approach, this branch initiated replacement therapy by the administration of the missing enzyme in hereditary diseases. The monitoring of the enzyme levels during and after this therapeutic procedure was done by the use of these radioactive substrates. It would be also of great interest to develop new methods which would allow relatively easily and inexpensively preparation of these compounds for the use of clinicians and for researchers who are not connected to a large research center. It is anticipated that L-glucosylceramide will be uniquely useful substance for exploring pathogenetic processes in animal analogues of Gaucher's disease.

Proposed Course: Work on this project continues in three major directions: 1. Glycolipids will be labeled by using the above mentioned techniques with ^{14}C and Tritium. 2. The approach using "minor synthetic modification" will be extended and used on lipids which were not prepared at all or not prepared by this technique. Also the replacement of the enzymatic oxidation will be explored. 3. Work will continue on the development of the technique: labeling by functional group exchange. 4. Work will continue on the synthesis of glycolipids which contain radioactive L carbohydrates instead of the naturally occurring D enantiomer. It is anticipated that these lipids will be uniquely useful for exploring pathogenic processes in glycolipidoses.

Publications:

1. Gal, A. E., Pentchev, P. G., Massey, J. M. and Brady, R. O.: L-Glucosylceramide. I. Synthesis, properties, and resistance to catabolism by glucocerebrosidase in vitro. Proc. Natl. Acad. Sci. USA 76: 3083-3086, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01480-13 DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Metabolism of Neurohumoral Transmitter Substances in Marine Animals		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div style="width: 60%;"> PI: E. G. Trams, Chief, Physiology and Metabolism Section, OTHER: N. Salem, Staff Fellow C. Lauter, Chemist J. Doherty, Toxicology Branch, EPA A. A. Benson, Biology Prof., Scripps Institute of Oceanography </div> <div style="width: 35%; text-align: right;"> DMN, NINCDS DMN, NINCDS DMN, NINCDS </div> </div>		
COOPERATING UNITS (if any) Mote Marine Lab., Sarasota, Florida Hazard Evaluation Division, Environmental Protection Agency, Washington, D.C. Scripps Institute of Oceanography, LaJolla, CA.		
LAB/BRANCH Developmental and Metabolic Neurology Branch, DMN		
SECTION Physiology and Metabolism		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD. 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.3</div>	PROFESSIONAL: <div style="text-align: center;">0.3</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 35%;"> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p> The purpose of this project is to explore the great variety and abundance of the <u>marine environment</u> for <u>molecular models of neurobiology</u>. In particular it was designed to investigate species or phenomena which display an amplification or simplification of human physiologic or pathologic metabolism. Most of the studies conducted during this period dealt with the biochemistry of plasma membrane preparations of axons derived from the walking leg of lobster (<u>Homarus</u>). The effect of a variety of pesticides on lobster axon plasma membrane ATPases was investigated for two reasons: 1. To relate pesticide toxicity, in particular neurotoxicity to their metabolic site of action and 2. To use pesticides as enzyme inhibitors for the purpose of discriminating between several closely related metabolic functions. </p>		

Project Description:

Objectives: To explore by experimentation in comparative biochemistry certain aspects of neurobiology that can be studied more easily and profitably in other than common laboratory species. Typical experimental models which have exploited the great variety or biochemical specialization of marine life forms are squid giant axon, torpedo electroplax and tetrodotoxin. Specifically, we use comparative studies to study the role of ecto-enzymes and adenylates in the modulation of cellular excitability.

Methods: A fractionation process was developed which allowed us to prepare plasma membranes from walking leg axons of the lobster (*Homarus americanus*). The membrane fraction was characterized by assaying for membrane marker enzymes. Enzyme assay were generally conducted with isotopic substrates. For comparative studies of ecto-enzyme activity, a variety of other species were also used, e.g., annelids (*glycera*), sea urchins (*lytechinus*), amoebae, yeasts and some lower vertebrates.

Major Findings:

Improvements in the method developed for the isolation of lobster axon plasma membranes gave a 10-20 fold purification over the starting homogenate. The membranes of lobster axons contained several ATPases, one of which related to the sodium pump, another was a Mg^{2+} activated ATPase, and another a Mn^{2+} ATPase which is probably a membrane ecto-enzyme. These enzymes could be identified according to their differential susceptibility to various pesticides, temperature/activity behaviours, and divalent cation requirements. It was shown that certain pesticides (kepone, DDT) produced a selective inhibition of ATPases at concentrations which suggested that these compounds may act in vivo as neurotoxins through ATPase inhibition in some cases. The Mn^{2+} -activated ATPase was stimulated by Mn^{2+} concentrations which are similar to those found in vivo and markedly inhibited by higher concentrations of this ion. This suggests that the neurotoxic effects of Mn^{2+} (for instance as observed in manganese mine workers) might be related to an inhibition of this enzyme. As an extension of this work, ecto-enzyme activity in synaptosomes and synaptic vesicles was studied. The results indicate that the membranes of synaptosomes and synaptic vesicles are derived from different portions of the neuron.

Proposed Course: We propose to extend our comparative studies on ecto-enzymes to several other species. It is possible that the very high ecto-ATPase activity in avian nucleated erythrocytes is related to metabolic regulation of the vascular bed; we propose to sample several other avian species to test this hypothesis. Further comparative studies will be made with unicellular organisms and with fishes, amphibians and reptiles. We propose to continue our investigations with crustacean axon membranes. Emphasis will be placed on critical experiments which relate to the role of polychlorinated hydrocarbon pesticides to neurotoxicity.

Publications:

1. Benson, A. A., Milhaud, G. M. and Trams, E. G.: Degenerative Processes in Spawning Pacific Salmon. In Shirmunsky, E. V. and Vernberg, F. J. (Eds.): Symp. Marine Biochem. and Physiol. XIV. Pacific Science Congress, Nakhoda and Kharborovsk, Acad. Sci. USSR (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01481-13 DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Studies on the Composition and Metabolism of Cellular Membranes		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div> PI: E. G. Trams, Chief, Physiology and Metabolism Section, OTHER: N. Salem, Staff Fellow C. Lauter, Chemist </div> <div style="text-align: right;"> DMN NINCDS DMN NINCDS DMN NINCDS </div> </div>		
COOPERATING UNITS (if any) Unit on Neurochemistry, CPB, NIMH Division of Food Science, Penn. State University		
LAB/BRANCH Developmental & Metabolic Neurology Branch		
SECTION Physiology and Metabolism		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD. 20205		
TOTAL MANYEARS: <div style="text-align: center;">2.9</div>	PROFESSIONAL: <div style="text-align: center;">2.9</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div><input type="checkbox"/> (a) HUMAN SUBJECTS</div> <div><input checked="" type="checkbox"/> (b) HUMAN TISSUES</div> <div><input type="checkbox"/> (c) NEITHER</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div><input type="checkbox"/> (a1) MINORS</div> <div><input type="checkbox"/> (a2) INTERVIEWS</div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p> The objective of this project is to elucidate the relationship between molecular composition and topographic arrangements of membrane building blocks with reference to plasma membrane function. Bioelectrogenesis, transport and many metabolic phenomena are based on the proper associations of membrane proteins and lipids. Membrane ecto-enzymes which are glycoproteins and require a lipophilic environment for optimal activity have been the primary object of these studies. Ecto-phosphoesterhydrolases appear to be a part of a regulatory system which modulates membrane permeability and excitability. Using covalently reacting chemical probes we have been able to achieve selective modifications of membrane ecto-enzymes. Ecto-ATPases and Ecto-5'-nucleotidases appear to be collocated with phosphatidylserine and phosphatidylethanolamine in the membrane. Neoplastic transformation of cells appears to be accompanied by an increase in ecto-ATPase activity and a decrease in ecto-5'-nucleotidase activity. Synaptosome-like vesicles which are enriched in these two ecto-enzymes are exfoliated from cells. These so-called <u>exosomes</u> appear to subserve an intercellular transport function. </p>		
29-DMNB/IRP		

Project Description:

Objectives: To elucidate the molecular composition and topographic arrangement of membrane building blocks and their role in defining the physiologic functions of the plasma membrane. Furthermore, to inquire if cell pathology in certain diseases of the CNS is associated with derangements of the function of such membrane components.

Methods: A variety of cultured cell lines derived from the CNS are employed. Established clones of neuroblastoma, glioma and several primary cultures are maintained in monolayer cultures. Enzyme activities on the monolayer cultures are primarily assayed by the use of radio-labeled substrates. In addition, High Performance Liquid Chromatography is used to follow transition of metabolic products through various compartments. Covalently reacting chemical probes are used to label cell surface constituents in situ and differential labeling is achieved by substrate protection of the respective enzymes. Lipids and proteins so labeled are analyzed after separation by classical techniques. Exosomes are harvested by ultracentrifugation or by filtration. Interaction of exosomes with cells is measured by labeling exosomes with radioisotopes.

Major Findings:

(1) A survey of ecto-enzyme activity in a variety of species showed that for the same cell type (e.g. erythrocytes) a great variation in ecto-enzyme activity existed. For instance, ecto-ATPase activity in avian erythrocytes is about one thousand-fold greater than in man. In a rat glioma cell line, with increasing dedifferentiation in continued culture, ecto-5'-nucleotidase activity virtually disappeared and ecto-ATPase activity may increase 20-30 fold. In this cell line the membrane permeability increase produced by ectopically applied ATP seems to be coupled to the presence of ecto-ATPase. A study of the divalent cation requirement of ecto-ATPase in several species has shown that in species which are low on the evolutionary scale, Mn^{2+} is the predominant activator, while in higher animals activation by Mg^{2+} or Ca^{2+} predominates. A comparison of ecto-ATPase and ecto-5'-nucleotidase activity in several normal and neoplastic cell lines indicated a preponderance of the former enzyme in transformed cells and a marked deletion of the latter. The correlation is suggestive but not absolute. We also have observed some significant changes in ecto-ATPase activity when rat or human erythrocytes are treated with bacterial endotoxins. The observations and some findings obtained in earlier experiments have led us to propose that ecto-ATPases serve a protective function in the vascular bed against the potent vaso-active effects of circulating adenylates. A hypothesis of the biochemical events leading to irreversible circulatory shock has been formulated.

(2) We have demonstrated that many cells exfoliate portions of their plasma membrane in the form of vesicles which measure 500 to 1000 nm in diameter. The portion of the plasma membrane which is shedded this way is not random, but consists of a selected part of the cell surface enriched in ecto-phosphoesterhydrolases. If these exosomes are super-

fused onto intact monolayer cultures, a time-dependent saturable uptake of exosome material takes place, indicating either fusion of exosomes with recipient cells or transfer of material contained within the exosomes.

(3) Studies on selective chemical modification of the cell surface made it possible to inactivate ecto-ATPase with nitrofluorobenzene derivatives without loss of ecto-5'-nucleotidase. Conversely, nucleotidase activity could be inactivated by an aromatic sulfonyl compound, leaving ecto-ATPase activity intact. Incubation of intact cells with chemical probes in the presence of the respective substrates protected the enzymes from inactivation. Experiments were designed which allowed for the selective labeling of presumptive active enzyme sites (or other portions significant for activity) with isotopic chemical probes. Our results showed that about one percent of the plasma membrane was occupied by ecto-ATPase in a glia-type cell (C6 clone). Using a chemical probe (TNBS) it was also found that replacement of inactivated ecto-5'-nucleotidase in the membrane was more consistent with de novo synthesis of the enzyme, rather than with insertion of preformed cytoplasmic enzyme into the membrane.

(4) In a collaborative study with Dr. P. Marangos (CPB-NIMH) we have purified and analyzed acid-soluble components of brain homogenates that appear to serve as putative ligands for the diazepam receptor. Our observations indicate that several of the proposed constituents of such brain extracts (i.e., inosine, hypoxanthine) occur in brain in significant portions only as preparation artifacts. At the present time we consider it probable that the material(s) that interact with the diazepam receptor are either peptides or as yet unidentified purine derivatives with high ligand activity.

Proposed Course

We will continue our investigations on the biological function of ecto-enzymes. It now seems probable that these biocatalysts have a multifunctional role and experiments will be designed to interrogate for probable functions in the appropriate model systems. Some of our studies shall address themselves to the question whether or not there is a significant correlation of ecto-ATPase or ecto-5'-nucleotidase with neoplasia, malignancy or differentiation. Another inquiry will be directed at the problem of the relationships between ectopic adenylates and ecto-enzymes in cellular excitability. Experiments are in progress in which selective chemical modification of plasma membrane enzymes will be used to study membranogenesis. Further investigations on the biological significance of exosome production are under way; in particular, their possible function as transport vehicles in vivo will be studied.

Significance

Our investigations have provided strong support for a role of adenylates as cellular excitability modifiers and for ecto-ATPase and ecto-5'-nucleotidase as regulators of this activity. The discovery of exfoliation of membrane vesicles (exosomes) suggests a new mode of intercellular communication or transport function.

Publications:

1. Butler, M., Salem, N., Hoss, W. and Spoonhower, J.: Raman spectral analysis of the 1300 cm^{-1} region for lipid and membrane studies. Chem. Phys. Lipids 29: 99-102, 1979.
2. Patton, S., Bogus, E. R., Stemberger, B. H. and Trams, E. G.: Antiserum to the milk fat globule membrane. Preparation and capacity to suppress milk secretion. Biochim. Biophys. Acta 597: 216-223, 1980.
3. Trams, E., Kaufmann, H. and Burnstock, G.: A proposal for the role of ecto-enzymes and adenylates in traumatic shock. J. Theor. Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01808-11 DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Glycoproteins of Myelin in Development and Disease		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div style="width: 65%;"> PI: R. H. Quarles, Chief, Myelin and Brain Development Section OTHER: R. O. Brady, Branch Chief D. A. Figlewicz, Guest Worker D. Johnson, Visiting Fellow S. Sato, Visiting Fellow </div> <div style="width: 30%; text-align: right;"> DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS </div> </div>		
COOPERATING UNITS (if any) Cellular Neuropathology Section, LNNS, NINCDS Veteran's Administration Hospital, Portland, Oregon		
LAB/BRANCH Developmental and Metabolic Neurology Branch, NINCDS		
SECTION Myelin and Brain Development		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md. 20205		
TOTAL MANYEARS: 6.1	PROFESSIONAL: 4.1	OTHER: 2.0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div style="width: 30%; text-align: center;"> <input checked="" type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%; text-align: center;"> <input type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) Central and peripheral <u>myelin</u> sheaths contain a high molecular weight <u>glycoprotein</u> that is selectively localized in the inner portion of the sheaths adjacent to the axon. This glycoprotein, referred to as the <u>myelin-associated glycoprotein (MAG)</u> , is specific for the nervous system and is present in greater amounts in the CNS than in the PNS. A major structural component of compact peripheral myelin (PO) is also glycosylated. Since membrane glycoproteins are known to be involved in recognition and cell-cell interactions, these myelin-related glycoproteins are being studied with regard to their likely roles in <u>glia-axon interactions</u> and myelin compaction. MAG undergoes a decrease in molecular weight during <u>development</u> that correlates well with myelin maturation. The chemical and immunological properties of the mature and immature forms of MAG are being studied. Since glycoproteins are well known to be <u>cell-surface antigens</u> and <u>receptors for viruses</u> , it is likely that MAG is involved in the putative auto-immune or viral aspects of <u>multiple sclerosis</u> or other <u>demyelinating diseases</u> . MAG is one of the earliest components to be lost in growing multiple sclerosis plaques, suggesting an important involvement in the pathogenesis of this disease		

Project Description:

Objectives: To investigate the biochemistry of cells of the nervous system with particular regard to glycoprotein components and their roles in myelination and demyelination. Other myelin and oligodendroglial proteins and lipids will also be examined with the ultimate objective of understanding the molecular mechanisms of myelin formation and breakdown. Emphasis will be placed on the major myelin associated glycoprotein and its role in demyelinating diseases such as multiple sclerosis.

Methods: Specific radioactive sugar precursors are used to label CNS and PNS glycoproteins. Myelin and other subcellular fractions are purified by differential centrifugation on sucrose gradients. Purified myelin is subfractionated into light, intermediate, and heavy fractions with different biochemical and morphological properties. Density gradient centrifugation is also used to isolate other oligodendroglial derived membranes (ODM). Enzyme markers are used to characterize the different subcellular fractions. The membrane-bound proteins and glycoproteins are fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Double label counting techniques are used for detecting the labelled glycoproteins on gels and revealing small differences between samples. Densitometric scanning of gels with Fast Green for proteins or periodic acid-Schiff reagent for glycoproteins is used for quantitation of individual protein components. Glycoproteins on gels are also detected by binding of radioactive lectins. Quantitation of individual lipids is carried out by thin-layer chromatographic separation and densitometric scanning of the TLC plates. Purification of the major myelin-associated glycoprotein involves solvent fractionation, preparative polyacrylamide gel electrophoresis, and column chromatographic techniques. Molecular fragments of the isolated glycoprotein are prepared by mild proteolytic or chemical cleavage. Gas liquid chromatography and colorimetric procedures are used for quantitation of individual sugars in glycoproteins. Rabbits are immunized with purified glycoproteins and antibodies are detected by Ouchterlony immunodiffusion or radioimmunoassay using (^3H) fucose-or ^{125}I -labeled antigen and anti-rabbit IgG serum.

Major Findings: The principal emphasis has continued to be on the isolation and characterization of the major myelin-associated glycoprotein (MAG). The protein is purified by the selective lithium diiodosalicylate (LIS)-phenol extraction procedure, followed by gel filtration on Sepharose CL-6B in the presence of sodium dodecyl sulfate (SDS). Use of SDS as detergent was found to give better resolution than the neutral ammonyx L0 described last year. High performance liquid chromatography (HPLC) is being explored as a faster and more effective procedure for the purification. Chemical analysis shows that rat brain MAG consists of one-third carbohydrate and two-thirds polypeptide. The polypeptide has an excess of acidic over basic amino acids, and a relatively high content of nonpolar amino acids. The larger immature rat MAG and human MAG are also being purified for chemical and immunological characterization.

Antibodies to MAG have now been produced in three rabbits, and the properties of the three antisera appear to be very similar. Experiments similar to those described in last year's report have been done with each antiserum and show that all are highly specific for MAG. An additional experiment showing the specificity of the antisera was the selective precipitation of (^3H) fucose-labeled MAG from whole brain homogenate by the double antibody procedure. Collaborative experiments with the Veterans Administration Hospital in Portland, Oregon, to test the effect of anti-MAG serum on myelinating cerebellar explants have now been completed. None of the three antisera exhibited demyelinating or myelination inhibition activity when applied to the explants. However, this negative finding can probably be explained by the inability of the antibodies to react with MAG in the intact membranes of the explant. Thus, we have found that each of the antisera is resistant to absorption by purified myelin or whole brain homogenate, and the antibodies only react with MAG after disruption of the membranes with detergent. The principle immunogenic site of MAG in rabbits appears to be inaccessible in the intact membranes.

Nevertheless, by appropriate utilization of detergents, the specific antisera have proven very useful for immunocytochemical studies and the developments of a radioimmunoassay for MAG. Immunocytochemical studies on MAG have continued in collaboration with the Section on Cellular Neuropathology, LNNS. The peroxidase-antiperoxidase method has been employed to examine MAG and myelin basic protein in a number of circumstances involving myelin pathology, including Jimpy mice, hexachlorophene intoxication, experimental allergic encephalomyelitis, and progressive multifocal leukoencephalopathy (PML). One of the most interesting findings was in PML, a human disease caused by a viral infection of oligodendrocytes, in which a decrease in MAG staining was found prior to the loss of myelin basic protein. This result is very similar to the early loss of MAG in developing multiple sclerosis plaques described in last year's report.

MAG was iodinated with Bolton-Hunter reagent for the development of a double antibody radioimmunoassay (RIA) capable of measuring 5 to 50 ng amounts of MAG in whole tissue homogenates. The antigenic sites of MAG are exposed by solubilizing the samples in 1% SDS, and the immune reaction is done in a mixture of 0.25% SDS and 0.25% Triton X-100. The Triton was found to prevent the inhibition of the immune reactions caused by SDS alone. Not only is this assay very sensitive, but it permits for the first time the measurement of MAG in crude samples without prior myelin purification and glycoprotein extraction. The RIA showed that the concentration of MAG is 0.3 μg per mg total protein in adult rat brain homogenate and 0.8 μg per mg protein in purified rat brain myelin. MAG was absent in newborn rat brain and increased as expected with the progression of myelination. MAG was not detected in non-neural tissues showing that MAG is a nervous system-specific protein. The RIA has also been applied to quantitate MAG in the tissue

culture systems (cerebellar explants and reaggregating cultures) that we are using to study myelination in vitro (see last years' report). Although the antiserum raised to rat MAG reacts with MAG from other species, its affinity varies from species to species leading to loss of quantitation when attempting to cross species lines with the RIA. However, in the case of human tissues, this difficulty was circumvented by using human MAG both for iodination and for the absolute standard in the assay. This simple adaptation of the RIA makes possible the measurement of MAG in human samples.

Last year, we reported that we had demonstrated (^3H)fucose-labeled MAG in rat sciatic nerve by employing the selective LIS-phenol extraction followed by immune precipitation with anti-MAG serum. We have now been able to visualize PNS MAG directly on SDS slab gels by electrophoresing the LIS-phenol extracts from a large number of sciatic nerves and staining with Coomassie Blue or periodic acid-Schiff reagents. The low amount of MAG obtained in the extracts suggested that PNS myelin sheaths have substantially less MAG than CNS sheaths. This was corroborated by RIA which showed that the concentration of MAG in rat sciatic nerve is only 15% of that in whole rat brain.

In the course of purifying MAG from post mortem human brain it was observed that MAG tended to be converted to a lower molecular weight derivative. The derivative has a molecular weight about 10,000 daltons less than intact MAG, contains about the same amount of carbohydrate as MAG, and reacts with anti-MAG sera. It was found that purified human myelin contains a neutral protease that converts MAG to the lower molecular weight derivative when the isolated myelin is incubated at 25° . The protease is activated by ammonium bicarbonate and other salts. The protease also degrades the basic protein in human myelin, but at a much slower rate. Over half of the MAG is converted to the derivative in a 30 min incubation, whereas it takes overnight incubation to degrade half of the basic protein. Further experiments showed that the protease was also present in purified rat myelin, but that the rat MAG was much less susceptible to its action. Overnight incubation of rat myelin was needed both for 50% conversion of MAG and for degradation of 50% of the basic protein. Thus, the protease is present in the purified myelin of both species, but the difference is that the human MAG is much more susceptible to its action.

Significance: The development of an RIA for MAG is a major breakthrough in the investigation of this glycoprotein, since it is now possible for the first time to measure MAG directly in whole tissue samples. This permits the quantitative comparison of MAG levels to those of other myelin constituents, such as basic protein, 2'3'-cyclic nucleotide phosphohydrolase, etc., in normal myelinating tissues, mutant mice exhibiting hypomyelination, and in demyelinating diseases. With regard to myelin formation, there is considerable evidence in the literature indicating that cell surface glycoproteins are involved in recognition and cell-cell interactions. The periaxonal localization of MAG in the

CNS suggests that it could be involved in oligodendroglial-axonal interactions during the course of myelinogenesis. Its similar periaxonal localization in the PNS suggests that it may also be involved in Schwann cell-axon interactions. Involvement of MAG in a common mechanism of glia-axon interaction in the two branches of the nervous system is consistent with recent evidence from other laboratories indicating that Schwann cells and oligodendrocytes are capable of myelinating axons from either the PNS or CNS. The higher level of MAG in the CNS may be needed for the more complex interaction of a single oligodendrocyte with many axons in contrast to the simpler one-to-one interaction of Schwann cells with axons in the PNS.

Demyelinating diseases such as multiple sclerosis are believed to involve autoimmune or viral processes. Since membrane glycoproteins are known to be cell surface antigens and receptors for viruses, it is not unlikely that MAG may be involved in the putative autoimmune or viral aspects of multiple sclerosis. Our finding that loss of immune staining for MAG is one of the earliest detectable events in the developing multiple sclerosis plaque strongly suggests an important role for this glycoprotein in the pathogenesis of the disease. There is currently a great deal of interest in the role of neutral proteases in demyelinating diseases, and basic protein is generally believed to be the molecule which is most susceptible to these enzymes. Our finding that human MAG is even more susceptible to a neutral protease may have considerable relevance concerning the biochemistry of demyelination. For these reasons, we believe that further elaboration of the chemical and immunological properties of MAG will increase our understanding of the molecular mechanisms underlying demyelination.

Proposed Course: Purification and characterization of MAG both from rat and human sources will continue. Proteolytic-and chemically-produced fragments of the molecule will be studied with the ultimate objective of determining its overall molecular structure. The chemistry of rat MAG can be related to the mechanism of myelinogenesis which is best studied in this species, whereas the chemistry of human MAG is important for understanding the molecular mechanisms of demyelinating diseases. The immunologically active sites in the MAG molecule will be determined. Since the anti-MAG sera prepared in rabbits reacts with sites that are inaccessible in intact membranes, monoclonal antibodies to MAG will be raised with the hope of obtaining antisera to more accessible portions of the molecule. Antisera of this type may permit further study of the function of this molecule.

The RIA for MAG will be used to compare the deposition of this glycoprotein with other myelin constituents in defined myelinating tracts. Similar studies will be done in mutant mice affected by abnormalities in myelin formation. Tunicamycin and other inhibitors of protein glycosylation will be used in myelinating tissue culture systems (explants or reaggregating cultures) to investigate the role of the

sugars in the function of this glycoprotein. Since the periaxonal localization of MAG strongly suggests an involvement in interactions with the axon, attempts will be made to isolate the putative axonal receptor for MAG from fractions enriched in axolemma.

The RIA for human MAG will be used to study the involvement of this glycoprotein in multiple sclerosis. We will measure the levels of MAG, basic protein and 2',3'-cyclic nucleotide phosphohydrolase in plaque, periplaque, and normal appearing white matter of multiple sclerosis tissue. This will put the early disappearance of MAG observed immunocytochemically on a quantitative basis. It will also serve to rule out inaccessibility as an explanation for the results of immune staining since the RIA is done in the presence of detergents. The cerebrospinal fluid and serum of multiple sclerosis patients will be tested for the presence of MAG or its fragments. Multiple sclerosis patients will also be tested for humoral or cellular immunity to MAG.

Publications:

1. Itoyama, Y., Sternberger, N. H., Webster, H. deF., Quarles, R. H., Cohen, S. R. and Richardson, E. P. Jr. Immunocytochemical observations on the distribution of myelin-associated glycoprotein and myelin basic protein in multiple sclerosis lesions. Ann. Neurol. 7: 167-177, 1980.
2. McIntyre, L. J., Quarles, R. H. and Brady, R. O.: Lectin binding proteins in central nervous system myelin: detection of glycoproteins of purified myelin polyacrylamide gels by (³H) concanavalin A binding. Biochem. J. 183: 205-212, 1979.
3. Quarles, R. H.: The biochemical and morphological heterogeneity of myelin and myelin-related membranes. In Kumar, S. (Ed.): Biochemistry of Brain, New York, Pergamon Press, 1980, pp. 81-102.
4. Quarles, R. H.: Glycoproteins from central and peripheral myelin. In Hashim, G. (Ed.) Myelin: Chemistry and Biology, New York, Alan Liss, in press.
5. Quarles, R. H., Johnson, D., Brady, R. O. and Sternberger, N. H.: Preparation and characterization of antisera to the myelin-associated glycoprotein, Neurochem. Res., in press.
6. Quarles, R. H., McIntyre, L. J. and Sternberger, N. H.: Glycoproteins and cell surface interactions during myelinogenesis. Soc. Neurosci. Symp. 4: 322-343, 1979.
7. Quarles, R. H., McIntyre, L. J. and Pasnak, C. F.: Lectin binding proteins in central nervous system myelin: Binding of glycoproteins in purified myelin to immobilized lectins. Biochem. J. 183: 213-221, 1979.

8. Trapp, B. D., McIntyre, L. J., Quarles, R. H., Sternberger, N. H. and Webster, H. deF.: Immunocytochemical localization of PNS myelin proteins: P2 protein is not a component of all PNS myelin sheaths. Proc. Natl. Acad. Sci., U.S.A. 7: 3552-3556, 1979.
9. Trapp, B. D., McIntyre, L. J., Quarles, R. H., Nonaka, G., Moser, A., Moser, H. W. and Webster, H. deF. Biochemical characterization of myelin isolated from the central nervous system of *Xenopus* tadpoles. J. Neurochem. 34: 1241-1246, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02162-06-DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Synthesis of Compounds Analogous to Glycolipids		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div> PI: A. E. Gal, Head, Neurochemical Methodology Section OTHER: F. J. Fash, Bio. Lab. Technician </div> <div style="text-align: right;"> DMN, NINCDS DMN, NINCDS </div> </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Neurochemical Methodology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.4</div>	PROFESSIONAL: <div style="text-align: center;">0.2</div>	OTHER: <div style="text-align: center;">0.2</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Glycolipid analogues</u> of sphingolipids were synthesized that yield a <u>chromo- genic moiety</u> on enzymatic hydrolysis. These compounds are used as reagents for the diagnosis of <u>Niemann-Pick disease</u> , <u>Gaucher's disease</u> and <u>Krabbe's disease</u> . The chromogenic analogues are also useful for the identification of <u>heterozygous carriers</u> of these disorders and for the <u>pre-natal diagnosis</u> of these diseases.		

Project Description:

Objectives: The compounds to be synthesized in the framework of this project are molecules similar to glycolipids which when cleaved enzymatically provide a chromophore useful for the diagnosis of lipid storage diseases and for the identification of heterozygous carriers.

Methods and Major Findings: Work is underway on the synthesis of a substrate for the chromogenic diagnosis of Farber's disease, a disorder characterized by a deficiency of ceramidase. This compound will be chemically related to 2-hexadecanoylamino-4-nitrophenyl phosphorylcholine (HNP). This substance resembles sphingomyelin but has a benzene ring instead of the aliphatic chain. Due to its nitrophenyl moiety, it yields an intense yellow color upon enzymatic cleavage. It is a reliable chromogenic substrate for assaying sphingomyelinase activity in diverse human tissue samples. It is used for the diagnosis of homozygotes and detection of heterozygous carriers of Niemann-Pick disease. This compound was synthesized by Calbiochem and Koch-Light and is commercially available from these manufacturers. We have developed a simplified synthesis of HNP using phosphorylcholine as the starting material. This improvement was realized because of the availability of free phosphorylcholine for which we developed a practical method of synthesis of HNP using phosphorylcholine as the starting material. This improvement was realized because of the availability of free phosphorylcholine for which we developed a practical method of synthesis. Based on the chemistry of HNP, research on non-radioactive sphingolipid substrates was extended to other lipidoses. Compounds were synthesized which could be used as substrates for measuring gluco- and galactocerebrosidase activities in tissue extracts. Thus, 2-hexadecanoylamino-4-nitrophenyl glucoside was shown to be a useful compound for the diagnosis of Gaucher's disease and 2-hexadecanoylamino-4-nitrophenyl galactoside can be used for the diagnosis of Krabbe's disease.

Significance: The new compounds were thoroughly tested and they have been found to be reliable for the diagnosis of lipid storage diseases. These findings constitute a major breakthrough because the previously required radiolabeled products are scarce, expensive, and not widely available. The chromogenic substances can be used and easily handled by practitioners and clinical chemists with no danger of radioactive contamination and they eliminate the necessity of costly and complex radioactive detection techniques.

Proposed Course: Based on the concept demonstrated by this project, additional compounds will be synthesized with chromophoric moieties for the detection of other enzyme deficiency disorders.

Publications:

1. Johnson, W. G., Gal, A. E., Miranda, A. F. and Pentchev, P. G.:
Diagnosis of adult Gaucher's disease. Use of a new chromogenic
substrate, 2-hexadecanoylamino-4-nitrophenyl- β -D-glucopyranoside
in cultured skin fibroblasts. Clin. Chim. Acta 102: 91-97, 1980.
2. Gal, A. E., Brady, R. O., Barranger, J. A. and Pentchev, P. G.
The diagnosis of Type A and Type B Niemann-Pick disease and
detection of carriers using leukocytes and a chromogenic analogue
of sphingomyelin. Clin. Chim. Acta, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02163-06 DMN
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Development of Special Analytical Methods and Preparative Techniques to Investigate the Etiology and Therapy of the Sphingolipidoses		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div> PI: A. E. Gal, Head, Neurochemical Methodology Section OTHER: F. J. Fash, Biol. Lab. Technician </div> <div style="text-align: right;"> DMN NINCDS DMN NINCDS </div> </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Neurochemical Methodology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.6</div>	PROFESSIONAL: <div style="text-align: center;">0.3</div>	OTHER: <div style="text-align: center;">0.3</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <u>New analytical techniques</u> were developed and used in enzymatic research and in clinical <u>investigations of lipidoses</u> . The lipid content in human tissues, the diagnosis of lipid storage diseases by <u>gas, thin-layer chromatography</u> and other techniques were studied at the microgram level. Also preparative work was done and used in connection with further synthetic work and for the <u>preparation of lipid standards</u> .		

Project Description:

Objectives: To develop techniques by which the separation and chemical analysis of biologic materials related to sphingolipidoses can be advanced. This work involves the following approaches: 1. Improvement of techniques leading to the separation of enzymes. 2. Development of biological materials.

Methods: The development of methods for the determination of lipids in small samples of biological materials of human origin such as erythrocytes, leukocytes, fibroblasts, serum, cerebrospinal fluid, urine or biopsy samples from kidney, liver and brain. The individual sphingolipids are present usually only in submicrogram quantities in these samples. For the separation of such lipids, thin layer and gas chromatographic procedures combined with column-liquid chromatography was used.

Quantitative evaluation was made by scanning of the thin-layer plates or by gas chromatography. Much work was done in areas not covered by existing literature references.

Major Findings: Gas chromatography of glucose originating from lipids could not be determined previously. This problem was solved by us. Also a new thin-layer chromatography system was developed which resulted in more reliable results using only small amounts of specimen. A novel technique was developed in which lipids present in the same sample (but not attacked by the exogenous enzyme) were used as internal standards. Improved analytical techniques showed practical results particularly in the studies related to replacement therapy of enzymes where the decrease of lipid levels in the liver and erythrocytes of patients was established and through these procedures an evaluation of the therapeutic effect of enzyme administration can be assessed.

Significance: The purification of the missing enzymes required for the therapy of the lipid storage diseases is a complex, tedious, and costly procedure. The identification of accumulated lipids in human tissues for the diagnosis and control of inherited lipid diseases is dependent on the sensitivity of the analytical techniques. The importance of accuracy in working with trace amounts of material in biological specimens necessitates improved techniques at the submicrogram level.

Proposed Course: Much more work has to be done in relation to the improvement of microanalytical procedures; for example, the ultra-microdetermination of aminosugars and sialic acid needs further development. Some of the existing methods are too complex and their simplification will be investigated. The application of other techniques including high speed (or pressure) liquid chromatography or the use of mass spectroscopy will be explored.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02366-02 DMN												
PERIOD COVERED October 1, 1979 through September 30, 1980														
TITLE OF PROJECT (80 characters or less) Regulation of Hormone-Responsive Adenylate Cyclase														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT														
<table style="width: 100%; border: none;"> <tr> <td style="width: 60%;">PI: P. H. Fishman, Research Biochemist</td> <td style="width: 10%;">DMN</td> <td style="width: 30%;">NINCDS</td> </tr> <tr> <td>OTHER: J. Hagmann, M.D., Visiting Fellow</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>S. Kassis, Ph.D., Visiting Fellow</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>J. B. Parent, Ph.D., Guest Worker</td> <td>DMN</td> <td>NINCDS</td> </tr> </table>			PI: P. H. Fishman, Research Biochemist	DMN	NINCDS	OTHER: J. Hagmann, M.D., Visiting Fellow	DMN	NINCDS	S. Kassis, Ph.D., Visiting Fellow	DMN	NINCDS	J. B. Parent, Ph.D., Guest Worker	DMN	NINCDS
PI: P. H. Fishman, Research Biochemist	DMN	NINCDS												
OTHER: J. Hagmann, M.D., Visiting Fellow	DMN	NINCDS												
S. Kassis, Ph.D., Visiting Fellow	DMN	NINCDS												
J. B. Parent, Ph.D., Guest Worker	DMN	NINCDS												
COOPERATING UNITS (if any) Laboratory of Molecular Biology, NINCDS Biological Psychiatry Branch, NIMH														
LAB/BRANCH Developmental & Metabolic Neurology Branch														
SECTION Enzymology and Genetics														
INSTITUTE AND LOCATION NINCDS, IRP, Bethesda, Md. 20205														
TOTAL MANYEARS: 2.75	PROFESSIONAL: 2.25	OTHER: 0.5												
CHECK APPROPRIATE BOX(ES)														
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER														
<input type="checkbox"/> (a1) MINDRS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) 1. Rat glioma C6 cells continuously cultured undergo a degeneration in their <u>catecholamine-responsive adenylate cyclase</u> system. <u>β-adrenergic receptor number</u> decreases and the cells do not accumulate <u>cyclic AMP</u> when exposed to β-agonists. Activity of catecholamine-stimulated <u>adenylate cyclase</u> is lower but not NaF-, GTP-, or <u>cholera toxin</u> -stimulated activities. 2. Prolonged exposure of C6 cells or <u>human fibroblasts</u> to β-agonists results in <u>desensitization</u> . The cells no longer respond to the agent but still respond to <u>cholera toxin</u> and, for the fibroblasts, to <u>prostaglandin E₁</u> . In contrast, fibroblasts exposed to <u>PGE₁</u> become desensitized not only to that agent but also to catecholamines and cholera toxin. Thus, β-agonists and <u>PGE₁</u> have different mechanisms of desensitization. 3. <u>Macrophages</u> exposed to cycloheximide lose their sensitivity to cholera toxin in parallel to inhibition of protein synthesis. The cells retain their toxin receptors and sensitivity to β-agonists. In addition, adenylate cyclase can still be activated by the A ₁ subunit of the toxin in cell membranes. As cycloheximide treatment blocks degradation of iodotoxin, the presence of a membrane component involved in <u>transmembrane translocation</u> of the toxin (or A ₁) is indicated by these results.														

Project Description:

Objectives: To investigate the molecular mechanisms involved in the regulation of hormone-responsive adenylate cyclase systems; to examine hormone-responsive adenylate cyclase during development and differentiation; to develop an overall model for this example of transmembrane signalling; to relate these findings to various metabolic disorders.

Methods: Guinea pig macrophages are induced in the peritoneal cavity with thioglycollate, removed and washed free of contaminating erythrocytes. The cells are placed in tissue culture dishes; and after 90 min. non-adhering cells are removed and the remaining macrophages are used immediately or cultured overnight. Rat glial C6 cells are an established cell line and are grown in monolayer culture on plastic dishes. Human diploid fibroblasts are from normal donors and are grown in monolayer culture as above.

Cells in situ are incubated with hormones and other effectors and levels of cyclic AMP are measured with a modified cyclic AMP protein binding assay. Adenylate cyclase activity in cell lysates or partially purified plasma membrane preparations is determined in the presence and absence of hormones and other effectors. Binding of hormones to intact cells and cell membranes is measured with radioactively labeled ligands. Bound ligand is separated from free by rapidly washing the monolayer cultures or by filtering the cells and membranes on small filters by means of a vacuum manifold. Specific binding is determined by correcting for radioligand bound in the presence of excess unlabeled ligand.

Major Findings:

A. Degeneration of the Beta-Adrenergic Responsive Adenylate Cyclase During Continuous Culture of Rat Glioma Cells

We compared low passage (LP) and high passage (HP) rat glioma C6 cells for differences in response to the beta-adrenergic agonist isoproterenol (ISO). C6LP cells exposed to ISO accumulated cyclic AMP (200-fold above basal) whereas C6HP (less than 2-fold). The latter cells also did not accumulate cyclic AMP when exposed to cholera toxin (CT). C6LP cells bound 3-fold more labeled beta-antagonist (dihydroalprenolol) and 2-fold more iodotoxin but ligand affinities were similar. Treatment of C6HP cells with G_{M1}, which increased the number of toxin receptors, did not increase their sensitivity to CT. ISO-stimulated adenylate cyclase activity was greater in membranes prepared from C6LP cells than from C6HP cells whereas NaF- and guanine nucleotide-stimulated activities were similar. In addition, adenylate cyclase was activated to a similar extent in membranes prepared from CT-treated LP and HP cells. We next determined that cyclic AMP phosphodiesterase activity was 3-fold higher in C6HP cells. When the latter cells were exposed to ISO or Ct in the presence of isobutylmethylxanthine, a potent phosphodiesterase inhibitor, they did accumulate some cyclic AMP but not as much as C6LP cells in the absence of the inhibitor. In addition, the rate

of cyclic AMP degradation in intact cells was greater in C6HP than in C6LP cells. Our results indicate that continual passage of rat glioma C6 cells in culture leads to a degeneration in the catecholamine-sensitive adenylate cyclase system; there is a decrease in beta-receptors and the efficiency of their coupling to the cyclase and there is an increase in the rate of cyclic AMP breakdown.

B. Mechanisms of Hormone-Mediated Desensitization

We compared rat glioma C6LP and C6HP cells for their ability to become refractory to stimulation after prolonged exposure to ISO. Both types of cells exhibited a time- and dose-dependent loss of ISO-responsiveness after treatment with the agonist. Following a 2-h exposure to ISO, ISO-stimulated cyclic AMP accumulation was reduced by 60-80% whereas binding of labeled dihydroalprenolol to beta-receptors was reduced only 10% without any change in ligand affinity. There was a corresponding large reduction in ISO-stimulated adenylate cyclase activity in membranes prepared from ISO-treated cells whereas NaF- and guanine nucleotide stimulated activities were unchanged. As C6LP cells accumulate 2-3 nmol of cyclic AMP per mg protein and C6HP less than 0.1 nmol when stimulated with ISO, these results indicated that ISO-induced desensitization is not mediated by cyclic AMP. This was confirmed by exposing C6HP cells to isobutylmethylxanthine which elevates cyclic AMP levels in these cells to the same extent as ISO alone. Cells treated with the former agent for 3 h did not become desensitized to ISO whereas cells treated with the latter did.

ISO-desensitized rat glioma C6 cells still accumulate cyclic AMP when treated with CT. When control and ISO-treated cells are exposed to CT, adenylate cyclase is activated to the same extent in membranes prepared from such cells even though activity is not stimulated by ISO in membranes from the ISO-treated cells. In addition, when membranes are incubated with the A₁ subunit of CT plus NAD, adenylate cyclase is activated irrespective of prior treatment of the cells with ISO. Human fibroblasts exposed to ISO for 1 h also become insensitive to a second challenge of agonist but still accumulate cyclic AMP when treated with CT or PGE₁. In contrast, fibroblasts exposed to PGE₁ for 1 hr become less responsive not only to PGE₁ but also to ISO and CT. Stimulation of adenylate cyclase activity by these agents as well as GTP in membranes prepared from PGE₁-treated cells also is reduced. These results indicate that catecholamine-induced desensitization involves a specific uncoupling of the beta-receptor from the adenylate cyclase without alterations of the regulatory or catalytic components of the cyclase system. Prostaglandin-induced desensitization appears to effect the regulatory component of the cyclase system as losses in ISO-, CT-, and GTP-stimulated activities are also observed.

C. Mechanism of Action of Cholera Toxin: Transmembrane Signaling

Cholera toxin (CT) binds to specific receptors (ganglioside GM_1) on the external side of plasma membrane and subsequently activates adenylate cyclase which is localized on the cytoplasmic side of the membrane. The mechanism of activation has been established in disrupted cells and involves the transfer of ADP-ribose from NAD to the guanine nucleotide binding component of the cyclase system, a reaction catalyzed by the A_1 subunit of CT. The intervening transmembrane steps between binding and activation, however, have not yet been elucidated. We find that macrophages exposed to cycloheximide no longer accumulate cyclic AMP when treated with CT. The loss of toxin sensitivity is dependent on the time of and concentration of cycloheximide in the medium and parallels inhibition of protein synthesis. Similar effects are observed with puromycin, another inhibitor of protein synthesis. The cycloheximide-treated cells still accumulate cyclic AMP when challenged with ISO. The cycloheximide-treated macrophages actually bind more iodotoxin than control cells; thus, loss of CT responsiveness is not due to loss of receptors. Membranes prepared from cycloheximide- and then CT-treated cells contain adenylate cyclase activity that can be stimulated by NaF, ISO and PGE_1 but the cyclase has not been activated by the toxin. However, when the membranes are incubated with NAD and the A_1 subunit of CT, adenylate cyclase is activated. Thus, cycloheximide does not appear to be affecting the guanine nucleotide regulatory component but some other step in toxin action. When control and cycloheximide-treated macrophages are incubated with iodotoxin, the toxin is rapidly degraded by the control but not the treated cells. Furthermore, when the cell-associated radioactivity is analyzed by SDS-polyacrylamide gel electrophoresis, small amounts of A_1 are detected in the control but not the cycloheximide-treated cells. We believe that cycloheximide is inhibiting the synthesis of a rapidly turning over membrane component that is required for translocation of CT (or its active subunit) across the plasma membrane.

Significance: These studies are providing information on the molecular mechanisms involved in the regulation of adenylate cyclase activity. As cells age in culture, their beta-adrenergic responsive adenylate cyclase system deteriorates. This may be a useful model for investigating effects of aging on adenylate cyclase. Desensitization is one process whereby cells can mediate their responsiveness to external stimuli such as hormones. The underlying mechanisms for hormonal-induced desensitization of adenylate cyclase activity are not known but our results indicate that different agents (catecholamines and prostaglandins) utilize different mechanisms. A cycloheximide-sensitive membrane component distinct from cholera toxin receptors and known components of adenylate cyclase is required to toxin action in intact cells; this component appears to be involved in the transmembrane signaling step.

Proposed Course: Excellent progress has been made in the second project. The project will be continued with emphasis placed on the mechanisms of hormonal desensitization and on the identification of membrane components involved in cholera toxin action. Other cellular models such as human diploid fibroblasts will be examined for age-related alterations of hormone-stimulated adenylate cyclase. In addition, other approaches to explore the molecular aspects of coupling of hormone receptors to adenylate cyclase will be attempted.

Publications:

1. Fishman, P. H.: Mechanism of Action of Cholera Toxin: Events on the Cell Surface. In Field, M., Fordtran, J. S. and Schultz, S. G. (Eds.): Secretory Diarrhea. Bethesda, Maryland, American Physiology Society, 1980, pp. 85-106.
2. Fishman, P. H.: Mechanism of action of cholera toxin: studies on the lag period. J. Membrane Biol. 54: 61-72, 1980.
3. Fishman, P. H. and Atikkan, E. E.: Mechanism of action of cholera toxin: effect of receptor density and multivalent binding on activation of adenylate cyclase. J. Membrane Biol. 54: 51-60, 1980.
4. Hagmann, J. and Fishman, P. H.: Modulation of adenylate cyclase in intact macrophages by microtubules: opposing actions of colchicine and chemotactic factor. J. Biol. Chem. 255: 2659-2662, 1980.
5. Parent, J. B., Tallman, J. F., Henneberry, R. C. and Fishman, P. H.: Appearance of beta-adrenergic receptors and catecholamine responsive adenylate cyclase activity during fusion of avian embryonic muscle cells. J. Biol. Chem. 255: in press, 1980.

Z01 NS 02433-01 DMN

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Models of Lysosomal Storage Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John A. Barranger, M.D., Ph.D.	Chief, Clinical Investigations and Therapeutics Section	DMN	NINCDS
Other:	George Constantopoulos, Ph.D.	Staff Biochemist	DMN	NINCDS
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	Nancy Krett	Guest Worker	DMN	NINCDS
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COOPERATING UNITS (if any)

Laboratory of Vision Research, NEI

LAB/BRANCH

Developmental and Metabolic Neurology Branch

SECTION

Clinical Investigations and Therapeutics

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS☒ (b) HUMAN TISSUES☐ (c) NEITHER☐ (a1) MINORS☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The possibility of enzyme replacement in lysosomal storage diseases requires extensive knowledge of the physiology and biochemistry of cells and their organelles, especially, the lysosome in the condition of storage. Animals treated with drugs (suramin, chloroquine) simulated lysosomal storage disease. Study of physiologic and biochemical aberrations in treated animals will define the milieu in which enzyme replacement can be attempted. Of particular interest is the ability of treated animals to incorporate infused enzymes, the function of receptors on plasma and lysosomal membranes, the ability of incorporated enzymes to interact with storage material, and the targeting of enzyme to cells and prolongation of their biologic activity in situ. Physiologic alterations are appropriately studied in animals, whereas, more detailed biochemical studies are more suitably conducted in strictly defined cell suspensions and cell cultures. Studies employing animal cells including macrophages, fibroblasts, hepatocytes, and Kupffer cells will be performed. Also, cultures of human fibroblasts and peripheral macrophages from control and disease states will be utilized.

Project Description:

Objectives: 1) Develop convenient animal models of lysosomal storage; 2) Evaluate morphologic and biochemical changes in treated animals including assay of lysosomal enzymes that are affected and accumulation of substances in lysosomes; 3) Study clearance of infused enzymes from circulation of treated animals; 4) Study incorporation and distribution of infused enzymes into organs of treated animals; 5) Study location, types, and numbers of receptors for lysosomal enzymes in treated animals compared to controls; 6) Evaluate efficiency of interaction of infused enzymes with lysosomal elements; 7) Increase delivery of enzymes to specific cell type and prolong biologic activity; 8) Study effects of complex glycolipids on normal and disease-state cells in culture; 9) Study receptors for glycolipids on membranes of cells in culture; 10) Evaluate effects of coculture of storage cells (e.g., Kupffer cells) with parenchymal cells (e.g., hepatocytes) to appraise interaction between cells; 11) Study location, types, and numbers of receptors for lysosomal enzymes on control and disease-state cells in culture; 12) Study effects of supplemental enzymes on storage of complex glycolipids by cells in culture and evaluate reversal of changes in those cells.

Methods Employed:

- 1) Treatment of rats with suramin and chloroquine by injection or oral route.
- 2) Light and electron microscopic examination of tissues.
- 3) Assay of lysosomal enzymes in tissue extracts and lysosomal fractions from treated animals.
- 4) Assays of mucopolysaccharides, glycolipids, and gangliosides in tissues and lysosomal fractions from treated rats.
- 5) Preparation of rats for clearance studies.
- 6) ¹²⁵I-labelling of lysosomal enzymes.
- 7) Isolation of purified fractions of rat hepatocytes and Kupffer cells and monolayer culture of these cells.
- 8) Radio-autography of tissues from enzyme supplemented animals and cells in culture.
- 9) Monolayer cultures of murine and human peripheral macrophages.
- 10) Radioligand-receptor analysis of glycoprotein enzyme receptors and glycolipid receptors of cells in suspension and culture.
- 11) Assay of lymphocyte activating factor, cytoplasmic and lysosomal enzymes released by cells in culture.

Major Findings:

- 1) Suramin treated animals demonstrate morphologic similarities to lysosomal storage disorders.
- 2) Mucopolysaccharides accumulate in tissues of suramin treated animals.
- 3) Gangliosides accumulate in tissues of suramin treated animals in a pattern similar to the mucopolysaccharidoses.

- 4) Certain lysosomal enzymes are non-competitively inhibited by suramin in vitro.
- 5) Suramin treated animals are a convenient model of mucopolysaccharidosis.
- 6) Non-activated (resident) murine macrophages in culture accumulate glucosylceramide (GL₁).
- 7) Other cells in culture (activated macrophages, fibroblasts, and lens epithelial cells) do not incorporate significant amounts of the glycolipid.
- 8) Other complex lipids (ganglioside GM₂, ceramide trihexoside, and sphingomyelin) are not incorporated significantly.
- 9) Macrophages that have accumulated GL₁ increase the production and release of lymphocyte activating factor (LAF) lysosomal enzymes, and cytoplasmic enzymes. The degree of these responses are directly related to the amount of lipid accumulated.

Significance to Bio-Medical Research and the Program of the Institute:

Inborn errors of metabolism including the lysosomal storage disorders are severely disabling or fatal diseases. Most have profound neurologic consequences. The studies conducted in this project are designed to better define the physiology and biochemistry of cells involved in the storage process. An understanding of the pathogenesis and alteration of cell function may assist in the intervention in the disease process with such techniques as enzyme replacement. As an example, the finding that of a number of cells, only macrophages (M ϕ) accumulate GL₁ and release lymphocyte activating factor, may be useful in monitoring the effects of enzyme supplemented to cells. More basically, the peculiar distribution of GL₁ in Gaucher's disease (i.e., only in M ϕ) may be the result of some special or specific property of the cell or glycolipid. Definition of that property may allow its manipulation to permit some measure of control of the disease. The observations that M ϕ release activators and lysosomal enzymes in response to storage of GL₁ gives a clue to understanding the multiple clinical problems of Gaucher patients. Particularly interesting is the injury of cells that are not involved in the storage of the lipid itself, but are obviously affected (e.g., parenchyma of liver, bone, and brain). This result may be mediated by the release of lysosomal enzymes and other toxins from M ϕ . Release of lymphocyte activating factor may explain the appearance of monoclonal and polyclonal gammopathies in these patients. Moreover, this immunological link could have bearing on the increased risk of malignancy in these patients. The finding concerning the difference between "resident" and "activated" M ϕ in their interaction with GL₁ provides a new parameter for defining the changes which accompany M ϕ activation. In addition to their role in defense mechanisms against infection or malignancy, the activated M ϕ are a major component of inflammation (particularly of the chronic type). These studies could provide a new approach to the characterization of the activated M ϕ .

The availability and appropriateness of animal and cell models of storage disease is obvious. Studies which cannot be done in humans because of risk or inability to control the variables can be accomplished in these systems.

Basic questions relating to the efficiency of enzyme replacement can be answered in the laboratory and the results used to tailor appropriate clinical trials. This will be especially true in studies of human disease macrophages.

Proposed Course of the Project: Areas that will be further investigated will concentrate on the human Gaucher macrophage ($M\phi$) in culture. Their ability to incorporate GL₁ will be evaluated particularly with respect to the specificity of uptake. Radio-ligand receptor analysis and autoradiographic techniques will be employed to determine if the monosaccharide portion of the glycolipid is responsible for the specificity. These techniques will also be employed to examine receptors on these cells and their subcellular elements for lysosomal enzymes. The aim of these studies is to determine the best possible ligand for the most efficient delivery and longest survival of supplemented enzymes in these cells. It is expected that maximizing these parameters will increase the efficiency of supplemented enzymes in reducing the accumulation of GL₁ and reversing the changes in $M\phi$ produced by the lipid accumulation.

The effects of corticosteroids on human Gaucher macrophages in culture will be evaluated. Parameters to be measured will be changes in endogenous enzyme concentration, changes in receptor-ligand binding, changes in glycolipid availability to supplemented enzymes and efficiency of supplemented enzyme to reduce GL₁ accumulation.

Other objectives to be pursued are the effects of coculture of glycolipid loaded murine Kupffer cells and hepatocytes.

Publications:

1. Rees, S., Cragg, B. G., Constantopoulos, G. and Brady, R. O.: Neuronal inclusions and attempts to identify them. In Kidman, A. D. and Tomkins, J. K. (Eds.): Muscle, Nerve and Brain Degeneration. New York, Elsevier North-Holland, 1979, pp. 212-221.
2. Gery, I. and Barranger, J. A.: Production and release of LAF by activated and resident macrophages. Fourth International Congress of Immunology, Paris, July, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02434-01 DMN								
PERIOD COVERED October 1, 1979 through September 30, 1980										
TITLE OF PROJECT (80 characters or less) Studies of Lysosomal Function: Receptor-Mediated Pinocytosis of Lysosomal Enzymes.										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 60%;"> PI: John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section </td> <td style="width: 40%; text-align: right;">DMN NINCDS</td> </tr> <tr> <td>Other: F. Scott Furbish, Ph.D. Staff Fellow</td> <td style="text-align: right;">DMN NINCDS</td> </tr> <tr> <td>Nancy Krett Guest Worker</td> <td style="text-align: right;">DMN NINCDS</td> </tr> <tr> <td>Roscoe O. Brady, M.D. Chief</td> <td style="text-align: right;">DMN NINCDS</td> </tr> </table>			PI: John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section	DMN NINCDS	Other: F. Scott Furbish, Ph.D. Staff Fellow	DMN NINCDS	Nancy Krett Guest Worker	DMN NINCDS	Roscoe O. Brady, M.D. Chief	DMN NINCDS
PI: John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section	DMN NINCDS									
Other: F. Scott Furbish, Ph.D. Staff Fellow	DMN NINCDS									
Nancy Krett Guest Worker	DMN NINCDS									
Roscoe O. Brady, M.D. Chief	DMN NINCDS									
COOPERATING UNITS (if any) None										
LAB/BRANCH Developmental and Metabolic Neurology Branch										
SECTION Clinical Investigations and Therapeutics										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205										
TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5								
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input checked="" type="checkbox"/> (b) HUMAN TISSUES</td> <td><input type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input checked="" type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS				
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input checked="" type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER								
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords) The uptake of <u>active glycoprotein lysosomal enzymes</u> occurs, in part, through the mechanism of <u>adsorptive pinocytosis</u> . <u>Receptors</u> for various parts of the enzyme molecule as <u>ligands</u> are present on the <u>plasma and organelle membranes</u> . It is the purpose of this project to study these receptors and utilize them for <u>targeting enzymes</u> to cells. These <u>binding capacities</u> may also play a role in <u>localizing glycoproteins</u> within the cell and thus may have a bearing on the <u>survival</u> of enzymes that have been incorporated into the cell. Thus, these studies are also directed toward <u>increasing the survival of exogenous enzymes</u> within certain <u>subcellular organelles</u> . The goal of these studies is to increase the interaction of exogenous enzyme with <u>stored material</u> in the cell and thereby increase the efficiency of <u>enzyme replacement</u> . These studies will be carried out initially in rats and in later phases in <u>human storage disease macrophages in culture</u> .										
57 DMNB/IRP										

Project Description:

Objectives: 1) Study the clearance of lysosomal enzymes from rat circulation using enzyme activity and ^{125}I -labelled enzymes; 2) Study the incorporation of infused enzymes and measure distribution to organs of the rat; 3) Study the distribution of infused enzymes to purified preparations of rat liver parenchymal and Kupffer cells; 4) Study survival time of infused lysosomal enzymes in rat liver and isolated hepatocytes and Kupffer cells; 5) Study survival time of infused enzymes in lysosomes of rat liver parenchymal and Kupffer cells; 6) Identify receptors on rat liver parenchymal and Kupffer cells for lysosomal enzymes; 7) Identify receptors on lysosomal and other cellular organelle membranes for lysosomal enzymes; 8) Modify lysosomal enzymes to increase delivery to either hepatocytes or Kupffer cells; 9) Use carbohydrate-enzyme conjugates to target lysosomal enzymes to either hepatocytes or Kupffer cells; 10) Modify lysosomal enzymes to increase survival in rat liver hepatocytes and Kupffer cell lysosomes; 11) Isolate and purify rat liver receptors for lysosomal enzymes; 12) Estimate the effect of steroid treatment on the number and types of receptors in rat liver; 13) Study uptake, survival and receptors for lysosomal enzymes in storage disease macrophages in culture; 14) Study effects of supplemental enzymes on reversing substrate accumulation and secondary alterations in storage disease macrophages; 15) Study efficiency of modified lysosomal enzymes in reversing substrate accumulation in storage disease macrophages; 16) Estimate effects of steroids on efficiency of lysosomal enzymes to reverse substrate accumulation in storage disease macrophages; 17) Study receptors on storage disease macrophages for substrates that are stored; 18) Estimate effect of steroids on the accumulation of substrate in storage disease macrophages.

Methods Employed:

1. Preparation of rats for clearance studies.
2. ^{125}I -labelling of lysosomal enzymes.
3. Radioligand-receptor analysis of glycoprotein enzyme receptors and glycolipid receptors on cells in suspension and culture.
4. Monolayer culture of human storage disease macrophages.
5. Radio-autography of cells and organelles.
6. Affinity chromatography of receptors for lysosomal enzymes.
7. Cyanoborohydride, carbodiimide, and other carbohydrate to protein conjugations.
8. Glycosidase modification of lysosomal enzymes.
9. Isolation of purified rat liver hepatocytes and Kupffer cells.

Major Findings:

1. Lysosomal glucocerebrosidase is cleared from the circulation in a biphasic manner. The majority of the enzyme is cleared with a half-time of approximately 15 minutes. A smaller amount has a considerably longer half-time in the circulation.

2. The more rapidly cleared form is subject to alteration of its clearance time by glycosidase treatment. The slowly cleared form is not.
3. Approximately 50% of the dose of enzyme appears in the liver.
4. The survival time in liver is approximately 8 hrs.
5. Of the enzyme recovered in liver, most of it is found in hepatocytes. Only about 2% can be recovered in non-parenchymal cells.
6. Sequential treatment of glucocerebrosidase with glycosidase results in the exposure of galactose, N-acetylglucosamine, and mannose moieties such that the monosaccharide is the predominant sugar of the glycoprotein and mediates its uptake. The uptake is saturable and can be competed with by specific monosaccharide terminal glycoproteins.
7. Receptors have been identified on hepatocytes for galactose terminal glucocerebrosidase and on non-parenchymal cells for N-acetylglucosamine and mannose terminal enzyme.
8. Utilization of the receptor on non-parenchymal cells for mannose terminal enzyme permits a 45-fold increase in activity of these cells whereas unmodified enzyme results in only a 7-fold increase in enzyme activity in these cells.
9. Fucose also plays a role in the uptake of glucocerebrosidase in hepatocytes. Treatment of the enzyme with fucosidase results in a significant decrease in uptake. No significant effect is seen on non-parenchymal cell uptake by fucosidase treatment.
10. Preliminary studies using glucocerebrosidase bound to agarose beads indicate that a binding protein exists in liver.
11. Studies of hexosaminidase indicate that the lysosomal survival time is identical in hepatocytes and non-parenchymal cells. However, the survival time is considerably longer than for glucocerebrosidase. Analysis of the properties of hexosaminidase that impart this longer survival may allow modification of glucocerebrosidase to increase its survival in lysosomes.

Significance to Bio-Medical Research and the Program of the Institute:

The lysosomal storage disorders are disabling or fatal diseases which frequently affect the nervous system. At present, there is no effective treatment for most of these diseases. The idea of enzyme replacement has been proposed and is promising. The studies performed in this project support the concept and confirm earlier observations that enzyme replacement is a possibility. The studies presented here are aimed at increasing the delivery of enzymes to cells and maximizing their catabolic activity on stored substrates. It is obvious that a considerable number of observations must be made to reach this goal,

if indeed, it is obtainable. It is encouraging that receptors for glucocerebrosidase can be utilized to increase delivery to Kupffer cells. The potential for targeting even greater amounts of enzyme may have been uncovered by these observations. This is fortunate as the Kupffer cells and other similar reticuloendothelial elements are the only cells in Gaucher's disease in which the storage material, glucosylceramide, is found. The results obtained with hexosaminidase may provide useful information which will lead to extending the survival time of other enzymes, such as glucocerebrosidase. Studies of human macrophages obtained from patients with storage disease should facilitate a number of observations in an experimental milieu which can be well controlled and should be pertinent to the actual disease. Results of these experiments should permit tailoring of clinical trials of enzyme replacement such that they will be efficient, conservative of enzyme, and clearly interpretable.

Proposed Course of the Project: Future work will concentrate on characterizing receptors on the plasma and organelle membranes of various cells, particularly the human macrophage in culture. Methods will be explored to increase interaction of supplemented enzymes with storage material.

Other modalities for increasing enzyme uptake and efficiency such as taking advantage of natural properties of the molecule (e.g., hydrobicity and charge) will be examined. This will necessitate elucidation of the carbohydrate and amino acid composition of the molecules and making appropriate comparisons between molecules that behave differently at the surface of plasma and organelle membranes.

Publications:

1. Kusiak, J. W. and Barranger, J. A.: 125 Iodine labeling of β -hexosaminidase A without modifying its properties. Clin. Chim. Acta 97: 155-158, 1979.
2. Brady, R. O., Barranger, J. A., Gal, A. E., Pentchev, P. G., Furbish, F. S. and Kusiak, J. W.: Treatment of lipidoses by enzyme infusion. In Lowden, J. A. and Callahan, J. W. (Eds.): Lysosomes and Lysosomal Storage Diseases. New York, Raven Press, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02435-01 DMN
PERIOD COVERED <p style="text-align: center;">October 1, 1979 through September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Studies On The Mechanism Of Pathogenesis Of The Mucopolysaccharidoses.</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI Other:	George Constantopoulos, Ph.D. Roscoe O. Brady, M.D. John A. Barranger, M.D., Ph.D. Investigations and Therapeutics Section Sandra Rees, Ph.D.	Research Biochemist Chief Chief, Clinical Dept. Physiology DMN NINCDS DMN NINCDS DMN NINCDS Monash Univ.
COOPERATING UNITS (if any) <p style="text-align: center;">Department of Physiology, Monash University, Clayton, Victoria, 3168, Australia.</p>		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Clinical Investigations and Therapeutics		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">1.5</div>	PROFESSIONAL: <div style="text-align: center;">1.5</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input checked="" type="checkbox"/> (a1) MINORS </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p> The primary objective of this project is the study of the <u>mechanism of brain involvement and mental retardation</u> in the <u>patients with mucopolysaccharidoses (MPS)</u>. We are using a <u>comparative approach</u>. For this purpose, whole brain, brain gray and white matter, brain cells and other fractions and tissues from patients with <u>MPS IH, MPS IS</u> (this patient was of normal intelligence), <u>MPS II, MPS IIIA, and MPS IIIB</u> and from non-neurological patients are examined for <u>glycosaminoglycans, sphingolipids and lysosomal enzymes</u>. To complement the studies with human subjects, an <u>animal model of mucopolysaccharidosis</u> is being developed. The drug suramin given intravenously and intracerebrally to <u>rats</u> inhibits <u>enzymes of glycosaminoglycan degradation</u> and causes <u>accumulation of glycosaminoglycans and sphingolipids</u> in the tissues. </p>		

Project Description:

Objectives: The mucopolysaccharidoses, like all the other heritable disorders can be considered experiments of nature. Because of the ubiquitous distribution of glycosaminoglycans in the human body, the lack of enzyme(s) required for their degradation affects many organs and functions. Our objective was to study the mechanism of pathogenesis of the various abnormalities characterizing the patients with MPS, with emphasis on the mechanism of brain involvement. For this purpose we try to correlate the chemical structure(s) of the involved compounds with the observed dysfunction (function). Because of the limitations arising from the lack of human material and of the requirement to modify "the experiment" at will, we are also trying to develop an animal model of MPS. We have administered the trypanocidal drug suramin, intravenously and intracerebrally, to rats. It seems that the suramin-treated rat may provide a useful animal model for the study of MPS.

Patient Material: Tissues were obtained at autopsy from 10 patients with MPS and from several non-neurological patients. Five had MPS IH, one had MPS IS, one had MPS II, one had MPS IIIA and two had MPS IIIB.

Hooded rats of different ages were used for the development of the experimental model.

Methods Employed:

- 1) Tissues were obtained at autopsy, the white and gray matter and the leptomeninges were carefully dissected and portions of the tissues were used for the isolation of neuronal perikarya, astroglia oligodendroglia and other brain fractions.
- 2) In hooded rats, suramin (250 and 500 mg/kg) was administered intravenously. In other experiments suramin was given intracerebrally (100 to 250 μ g in multiple injections) to rats.
- 3) Glycosaminoglycan content, composition, and molecular weight distribution, lipids including sphingolipids and the activities of a number of lysosomal enzymes including 8 required for the degradation of the glycosaminoglycans, were determined.

Major Findings:

For the past 10 years we have examined the biochemical changes found in the urine, blood and CSF of about 50 patients with various types of MPS. This was followed by a study of the neurochemistry of whole brain, gray and white matter, leptomeninges and blood vessels and dura mater of the patients who died. In spite of the biochemical findings - that is the accumulation of glycosaminoglycans in the brains of patients with MPS IH, MPS II, MPS IIIA and MPS IIIB - histological, histochemical, and electron microscopic examination of the brains showed that the neuronal cytoplasm was distended by excessive amounts of lipid staining material.

1. In order to understand the pathogenetic processes leading to excessive storage of lipid-staining material in the neurons, we have isolated in bulk neuronal perikarya, astroglia, axons and other fractions from patients with MPS IH, MPS IS, MPS II and MPS IIIA and from normal control brains. Glycosaminoglycans and sphingolipid were measured for the first time in normal human brains. Glycosaminoglycans, as μg per mg protein were: 2.2 in neuronal perikarya, 2.0 in astroglia, 2.1 in oligodendroglia, 3.3 in neuropile and 0.8 in myelinated axons. Chondroitin sulfates constituted more than one half of the total glycosaminoglycan. Hyaluronic acid, heparan sulfate, and dermatan sulfate were also present in all cells and fractions. There was a 4- to 6-fold increase in the concentration of total GAG in the neuronal perikarya of patients with MPS IH, II, and IIIA. The increased GAG were heparan sulfate in MPS IIIA, and dermatan sulfate plus heparan sulfate in MPS IH and II. The concentration of the gangliosides GM_2 , GM_3 and GD_3 together amounted to 65% of the total gangliosides of neurons, indicating changes of the same magnitude seen in the gangliosidoses. All these patients exhibited mental retardation. In contrast, the concentration of GAG and sphingolipids in the brain of the patient with MPS IS was normal. This patient was of normal intelligence. The results suggest that the partially degraded heparan sulfate and perhaps the dermatan sulfate, which accumulate in the brain of patients with MPS IH, II, IIIA and IIIB may inhibit catabolic enzymes of various sphingolipids. In turn the accumulation of sphingolipids in the neurons may account, at least in part, for the occurrence of neurological signs and progressive dementia in patients with MPS IH, II, IIIA and IIIB.
2. Intravenous administration of suramin, 500 mg/kg to 2 month-old rats causes a 5- to 8-fold increase of GAG concentration of the liver within 10 days and a 6-fold increase in urinary GAG excretion. The excessive GAG consist of heparan sulfate and dermatan sulfate. Intracerebral injection of 250 μg suramin causes a GAG increase and also a larger increase of ganglioside GM_2 , GM_3 and GD_3 concentration in the treated region of the brain. The activities of the lysosomal enzymes iduronate sulfatase, β -glucuronidase and hyaluronidase in the liver of the suramin-treated rats were consistently decreased while the activities of the other enzymes were increased. The activity of iduronate sulfatase was completely inhibited in vitro by a $5 \times 10^{-5}\text{M}$ or higher concentration of suramin. The activity of β -glucuronidase was also strongly inhibited by low concentrations of suramin but the inhibition was partially cancelled at higher concentrations of the drug. The inhibition of both enzymes by suramin was non-competitive. The suramin-treated rat may provide a useful experimental animal model of mucopolysaccharidosis.

Significance to Bio-Medical Research and the Program of the Institute:

The mucopolysaccharidoses are inborn errors of metabolism resulting in lysosomal storage disease. The pathogenesis of these disorders is poorly understood. If inroads to therapy are to be made, a more complete knowledge of the mechanisms of disease will be required. To this end, these studies have contributed additional observations to the molecular biology and pathology of the mucopolysaccharidoses. As data of this sort accumulate, a better understanding of the pathogenesis will result.

The studies of the suramin model will assist in the understanding of the pathogenesis and describing secondary changes. Data indicates that accumulation of the drug and the attendant changes in MPS and sphingolipids may be reversible. If this is correct, reversibility of lesion maybe possible in this model and, perhaps, in the true disease state. These aspects may have profound implications for therapeutic endeavors in these disorders.

Proposed Course of the Project: We expect to extend our studies to the remaining types of MPS and to isolate lysosomes from tissues of the patients in order to study the biochemical changes in these primary loci of the disorders. Regarding the suramin animal model, we propose to (i) study the time and dose response of the drug; (ii) To examine the effect of suramin on other organs such as kidney and spleen and other lysosomal enzymes such as the enzymes of sphingolipid degradation; and (iii) to study the reversibility of the biochemical changes caused by suramin.

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neuropathology and Neuroanatomical Sciences
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 to September 30, 1980
Laboratory of Neuropathology and Neuroanatomical Sciences, IRP
National Institute of Neurological and Communicative
Disorders and Stroke

Igor Klatzo, Chief

The LNNS has continued expanding in the scope of its research projects and in the presentation of its findings at numerous national and international meetings.

The Section on Cerebrovascular Pathology has further documented the interrelationships between the resolution of vasogenic brain edema (VBE) and the intracellular uptake of extravasated serum by the glial elements. A new hypothesis formulated on these findings was presented at the Symposium on Brain Edema in Berlin, September 1979. In the past year, these studies have been extended to include the observations on cerebral embolism by microspheres, occlusion of the medial cerebral artery (MCA) and chronic hypertension in the stroke-prone, spontaneously hypertensive rats (SPRSH). The parameters for regional correlations included: 1) estimation of water content in the tissue by specific gravity measurements, 2) evaluation of cerebrovascular permeability by immunocytochemical method (PAP) designed for demonstration of extravasated serum proteins, by application of radioactive and fluorescent blood-brain barrier (BBB) tracers, and 3) estimation of cerebral blood flow (CBF) by autoradiographic and microsphere methods.

The main findings from these studies were: 1) The direct relationship between the presence of extravasated serum proteins in the extracellular spaces and the VBE was confirmed in microembolization and middle cerebral artery occlusion experiments; 2) in cerebral ischemia produced by both methods the development of vasogenic component of the ischemic brain edema could be clearly outlined by specific gravity measurements and the PAP method; 3) in hypertensive (SPRSH) rats, a chronic intermittent leakage of the BBB to proteins resulted in extensive spreading of extravasated proteins through the white matter, as demonstrated by PAP, whereas testing of the BBB with the conventional tracers revealed only minimal or no changes in cerebrovascular permeability. There was also a striking localization of serum proteins in the cytoplasm of seemingly well-preserved neurons in the adjacent gray matter regions.

In collaboration with the Neuroradiology and Computed Tomography Section, Surgical Neurology Branch, serial measurements of CT attenuation were compared with specific gravity measurements in the cold injury VBE produced in the monkeys. In these studies, the drop in specific gravity measured in fresh, unfixed specimens from both the edematous and normal hemispheres corresponded closely to the decrease in CT attenuation values obtained before the sacrifice. Thus, the quantitative correlations between CT and specific gravity measurements demonstrated the degree of sensitivity and usefulness of CT in following the progression and resolution of the VBE.

In another investigation the formation of astrocytic fibrils was observed in cerebral ischemia using the PAP method designed for demonstration of glial fibrillary acidic protein (GFAP) by specific antibody. In cerebral micro-embolization, a striking astrocytic reaction expressed in formation of dense GFAP fibrils was observed as early as 10 minutes following intracarotid injection of microspheres. The MCA occlusion experiments revealed that, in animals sacrificed when areas of serum protein exudation were just beginning to appear (approximately 6 hrs after occlusion), the extensive and conspicuous areas of GFAP reaction in astrocytes could be demonstrated in the brain regions affected by ischemia but not showing evidence of any serum protein extravasation. The GFAP reaction, in view of its importance as representing the most common pathological change in the brain (astrocytic gliosis), is being further investigated with regard to elucidation of the possible mechanisms involved.

For the past year, the Section on Neurocytobiology has continued to investigate: I. The altered blood-brain barrier (BBB) and metabolic state in complete ischemia, particularly the prevention and therapy of experimental cerebral ischemia using central nervous system depressants. II. The biochemical, histochemical and morphological properties related to the transport and/or metabolism of cerebral capillaries, pia-arachnoid and neurons in vivo and in vitro which include studies in tissue culture.

I. 1) The continuous effort in evaluating the beneficial influence of the naturally occurring CNS depressants [γ -butyrolactone (GBL) and γ -hydroxybutyrate (GHB)] on cerebral ischemia has been focused on correlating their effect with that of the barbiturates [pentobarbital (Pb)] in ischemic cerebral edema. Both the endogenous (GBL and GHB) and the exogenous (Pb) CNS depressants reduced the brain edema as was manifested by the amelioration of the water content in the brain (measured by the tissue specific gravity) and the BBB leakage to sucrose and the monoamines. However, GBL and GHB were found to be more effective than the Pb treatment, irrespective of whether they were injected prior to or after the induction of the ischemic brain edema. These findings are in agreement with those of the greater survival rate of animals and the faster recovery of energy and carbohydrate metabolites in the brains of gerbils treated with GBL or GHB than with Pb. The mechanisms of action in the GBL and GHB is still unclear but under investigation.

2) A. In the study of the pathogenesis of ischemic cerebral edema, the biochemical events were correlated with the water content in the brain. These investigations have shown that a) decrease in the activities of ATPase system and the serotonin level increases the water content, b) enzymes of the main energy producing system are activated with the increase of water content, c) biogenic amines, the GABA system and high energy phosphate metabolites show no correlation to the cerebral edema. Thus, many factors as well as their possible interaction are involved in the development and progression of ischemic cerebral edema.

B. The correlation of the selective BBB changes with the tissue water content in bilateral cerebral ischemia suggests that the secondary increase in the tissue water coincides with the BBB leakage of serotonin but not with albumin as it is the case in edema induced by unilateral carotid artery occlusion.

II. 1) Further studies connected with the elucidation of the mechanism responsible for the normally limited BBB passage of the monoamines indicate that the microvessels have the capability for 5-hydroxytryptamine (5-HT) and norepinephrine (NE) uptake and metabolism as well as the inability to store their metabolites. The turnover rate for ^{14}C labeled 5-HT and ^3H NE was found to be 189 nmoles/mgP/30minutes, and 94 pmoles/mgP/30 minutes, respectively. Anoxia reduces markedly the capillary turnover of both substrates. Thus, the cerebral capillaries are most likely responsible for regulating the inflow and outflow of 5-HT and NE by inactivating the amines which can be altered under pathological conditions.

2) The characteristic capillary inability of monoamine storage and scarce pinocytotic activity found in the established endothelial cultures derived from dissociated cells of isolated cerebral microvessels provide an excellent model for the study of pathomechanisms of BBB disturbance occurring in various cerebral disorders.

3) The pia-arachnoid membrane as a constituent of BBB has been cultivated and evaluated in regard to the monoamines uptake and pinocytotic activity which are restricted by the BBB in vivo. Both cell types (endothelium and pia) of the pia-arachnoid membrane showed a limited uptake of catecholamines and serotonin. L-dopa was taken up by the endothelial but not by the pial cells while the reverse situation was observed in regard to the pinocytotic activity.

The diverse cellular properties of pia arachnoid provide a very useful in vitro model for the study of the blood-cerebrospinal fluid barrier and the BBB relationship under physiological and pathological circumstances.

The Section on Experimental Neuropathology has been engaged in an analysis of material prepared according to two principles for an improved preservation of neuronal glycogen which has often been difficult to achieve with earlier techniques.

A) One principle consists of two steps, namely the addition of iodoacetic acid to each solution (saline solution for removal of blood; Bouin's solution for fixation; ethyl alcohol for dehydration in situ) used for perfusion fixation and dehydration of the brain in order to reduce the rate of hydrolysis of glycogen before fixation is accomplished, and the injection of epinephrine intracardially for exclusive flow of the solutions to the brain. As an expression of an improved preservation, glycogen was demonstrated (1) in cortical neurons in which it had not previously been detected, (2) in cerebellar

Purkinje cells, in which it had not previously been discerned except under unusual circumstances, and (3) diffusely through the Purkinje cell perikarya as in other cells prepared by the Altmann-Gersch freeze-drying technique for preservation of the normal distribution of glycogen; and (4) was found to be more intensely stained in motor neuron Nissl bodies than with other techniques. This method permits a more accurate assessment of the distribution of neuronal glycogen in normal and experimental animals, which is crucial for a correct interpretation of the effect of an experimental procedure on carbohydrate metabolism or on glycogen metabolism more specifically.

The significance of an improved method of fixation of glycogen was tested in three experimental situations: (I) When the animals were treated with cortisone, the stainability of neuronal glycogen was more intense than previously experienced by fixation with Bouin's solution without iodoacetic acid. Cells like the Purkinje cells were disclosed to have accumulated glycogen throughout their perikarya; however, this did not prevent the alcohol-induced polarization from taking place in cells situated near the surface. (II) After axotomy, there was a distinct loss of glycogen which, however, was less intense than observed previously with routine perfusion fixation. The cells with reduced amounts of glycogen had a faint pink color. A postaxotomy loss of glycogen was also detected if only a smaller branch of the facial nerve was transected. Under these circumstances scattered uninvolved neurons would display a normal intense glycogen staining. (III) In the 2-week-old rabbit, the depression of glycogen staining was noticeable already 1 day after axotomy and not 2 days postaxotomy as in the adult animal. These differences are consistent with differences in moment when disintegration of Nissl bodies or dispersal of ribosomes takes place centrally in the young rabbits and peripherally in the adults.

Alcohol-induced polarization of perikaryal glycogen was not demonstrated in the deeply situated Purkinje cells but did occur in those near the surface.

With the development of a method for an improved stainability of neuronal glycogen, glycogen was demonstrable in the cerebellar mossy fibers and a zonal pattern in distribution of the glycogen-filled fibers emerged. In order to verify the origin of these fibers, hemisections of the spinal cord were performed on the rabbit. The results of these operations, which affect the content of glycogen in these fibers, are awaiting the histologic preparation.

B) Another principle is to add DMSO to the three previously mentioned perfusates for the purpose of accelerating passage of the fixative across the vascular and cellular membranes. Two aims were achieved, namely to prevent the development of glycogen polarization induced by alcohol in the superficial Purkinje cells and to obtain a more intense stainability of neuronal glycogen as well as of glycogen in oligodendrocytes and in microglial cells. The limitation of stainability to a narrow zone around the blood vessels is attributed to a limited diffusion rate of the DMSO. The haphazard distribution of the intensely red-stained cells is ascribed to one of two possibilities, namely either that only cells in metabolic state of increased glycogen storage were fixed or that an irregular flow of the perfusates permitted the fixative to

reach only scattered groups of cells. The development of myriads of pericapillary foci with shrunken neurons and an unusual, not previously described, segmental atrophy of dendrons indicates that DMSO as used in this technique may not be completely innocuous, but rather toxic to the central nervous system. This is the more intriguing since this drug has recently been reintroduced in clinical therapy, although with restricted use. It should be noted that the epinephrine injected intracardially before perfusion so as to achieve an exclusive flow of the perfusates to the brain may have had a potentiating effect.

In order to determine what effect iodoacetic acid may have on the formation of the intensely red, dimedone-PAS-stained neurons, a Netherlands dwarf rabbit was perfused with solutions mixed with DMSO but without the addition of iodoacetic acid. The paraffin-embedded material is awaiting histologic preparation.

In the Section on Neurocytology, studies on regeneration of peripheral neurons has been extended to central neurons. The characterization of particle assemblies within astrocytic membranes and the mechanisms whereby protein and ions cross cerebral blood vessels continue to be explored.

We have now learned that allografts of superior cervical ganglion (SCG), transplanted to the intact ventricular and pial surfaces, have not been rejected as late as two years after the transplantation. Although the SCG fragments decrease in size with time, both myelinated and unmyelinated neuronal processes wrapped in Schwann cells, persist. These observations demonstrate that the undisturbed brain surface can support regenerating neurons for at least two-thirds of the animal's life.

The reaction of the undisturbed brain surface differs with the cerebral region. The external granule cells and associated neurons of the cerebellum migrate anomalously, out of the cerebellum toward the SCG transplant so as to form ectopic glomeruli. Nests of Schwann cells from the transplant invade the cerebellar cortex and compete with astroglia in the partial envelopment of in situ synaptic glomeruli. When the transplant is placed over the area postrema, bridges, consisting primarily of reactive astrocytes with some myelinated axons and synaptic terminals, emanate from the medulla. Neurons of the area postrema do not migrate. Within the SCG transplant, regenerating ganglion cells in the CSF can produce and store noradrenaline (NA), detected cytochemically. Small (40-50 nm) and large (80 nm) NA-storage vesicles occupy discrete areas in the transplanted ganglion cells and appear to increase over a 5-month period. Numerous storage vesicles also lie within axons and growing tips, indicating that axoplasmic transport is being performed. Autonomic ganglia, transposed to CSF surfaces, not only regenerate but can synthesize and transport their neurotransmitter.

Our interest in regeneration of central neurons is its specificity and, ultimately, ways of influencing it. The motoneurons of the frog spinal cord have two afferent inputs: one from the medulla which end chiefly on motoneuron somata and proximal dendrites, and a segmental one from dorsal root ganglia,

ending on the distal dendrites of the same motoneurons. If one afferent source is removed, can the other take its place at the vacated synaptic site? Recently initiated experiments include cord transection at mid-thoracic levels in order to remove the medullary input onto lumbar motoneurons. We have developed means of applying HRP to the central dorsal root so as to label its intraspinal projections. Eight weeks after cord transection, a small cellular bridge connects the rostral and caudal stumps of the spinal cord. The next step is to see whether the labeled regenerating axons make synaptic contact with those dendrites that normally receive medullary afferents.

Our most recent attempts to elucidate the composition and function of the orthogonal aggregates of intramembranous particles belonging to astrocytes, has led, unexpectedly, to the observation that if the cultures are not fed every four days as prescribed but rather, only every 7th or 10th day, the astrocytes are less fusiform and more asteroid, and their assembly content increases to more normal numbers. Since leptomeninges are included in the preparation of the dissociated brain cells, the meninges might contribute a "maturation" factor to the medium. Accordingly, glial cultures were established with or without arachnoid - pial cells. No difference in glial cell-shape or assembly-content resulted, nor was there any change when the astrocytes were co-cultivated with cells of the adenohypophysis, which have been purported to liberate a glial proliferative substance. Although neurons are not supposed to survive explantation from 7-day-old rats under the conditions used here, we have very recently noted that a few cells, intermingled with the astrocytes, have neuron-specific enolase as detected immunocytochemically. These neurons might produce a glial "maturation" factor which would accumulate in the medium over time. The co-cultivation of astrocytes with certain types of neurons (e.g., SCG) is underway. There is some evidence that the distribution of assemblies may be influenced by the underlying cytoskeleton. After exposure to 10^{-7} M colchicine for 1 hour, some of the astrocyte membranes become crowded with assemblies so as to resemble the adult in situ membrane. The remarkable redistribution indicates an association of subunits with the cytoskeleton. This association will be explored in cold lesions to compare the arrangement of assemblies here with the highly ordered alignment in astrocytes reactive, in situ, to SCG transplants.

³⁺The mechanisms whereby horseradish peroxidase (HRP) and ionic lanthanum (La^{3+}) cross cerebral vessels during hyperosmotic stress are now being examined under conditions that should inhibit energy-requiring events. In 10 rats, the replacement of systemic blood with balanced salt solution to yield a brain core-temperature of either 35°C or 15°C has resulted in exudation of HRP in both groups. In living rats, the core temperature has been held at 25°C. If successful, the experiments should indicate whether the exudates are formed actively, i.e., by vesicle formation, or passively, i.e., by a flow through open, endothelial junctions.

The main interest of the Section on Functional Neuroanatomy has continued to be in understanding synaptic transmission and development. The rapid-freezing technique developed in this Section has been improved and applied to capture fleeting structural changes in functioning synapses. Since the temporal resolution of rapid freezing is less than 2 msec, as measured by a capacitance method developed here, the fate of synaptic vesicle membrane after vesicles fused with the synaptic plasmalemma could be seen. We found that the vesicle membrane is flattened out into the plasmalemma in less than 0.1 sec after transmitter release. Particulate components of the vesicle membrane then spread out and are collected a second later in particle islands incorporated into coated vesicles. The ultimate fate of these components of the vesicle membrane is to be reincorporated into synaptic vesicles. This finding of recycling extends our earlier work showing that local recycling of synaptic vesicles replaces those lost during synaptic activity.

Synaptic vesicles are so small and the initiation of exocytosis is so rapid that visualization of its initial stages has been elusive. In order to see this process in more detail, amebocytes from Limulus were frozen at various times after inducing them to secrete. These cells have large secretory granules which are secreted within a few seconds after exposure to endotoxin, so it was possible to see the beginning of exocytosis, a tiny hole in the plasmalemma which subsequently widens. This finding is of interest because it is incompatible with the current idea that exocytosis begins as a broad approximation between the secretory granule and the plasmalemma, which then thins and bursts. These new results require instead that a local disruption in the adjacent lipid bilayers be considered the initial event in exocytosis, at least in these cells. A second important feature of exocytosis is that the plasmalemma puckers in to contact the secretory granule just before exocytosis begins, and filaments are associated with this process. Thus, a contractile process may be associated with initiation of exocytosis. These studies have produced a clear picture of the membrane interactions leading to exocytosis, which we have shown is also the basis of neurotransmitter release.

The rapid freezing technique makes feasible a variety of other types of experiments. This technique was used to immobilize a calcium ion in neural tissues, so that subsequent cytochemical techniques could be used to fix calcium at its natural locations. The calcium is then detected with an electron probe x-ray spectrometer. In the synapse, the calcium which enters during prolonged electrical stimulation is sequestered in cisterns of endoplasmic reticulum. Where this calcium enters is also a subject of investigation. Counts of the membrane particles associated with active zones in the squid synapse have shown that, if these particles are the calcium channels, their conductance is in line with the conductances of other calcium channels. This concurrence suggests the conclusion that these particles are the calcium channels and that an intimate association between calcium influx and synaptic vesicle exocytosis is definitive of the synaptic active zone. These studies are producing a clear view of the dynamic aspects of the structures at neuromuscular synapses.

Synapses degenerating after nerve resection have also been investigated. Just at the point when the evolved potential disappears, the active zone particles, which our new evidence suggests are the calcium channels, also disappear. Spontaneous potentials, however, only disappear after the nerve terminal becomes engulfed by Schwann cells. Thus, the freeze fracture technique is revealing the fundamental membrane and cellular processes which underlie the failure of neuromuscular transmission which follows a nerve injury.

In the Section on Cellular Neuropathology, investigators are using immunocytochemical techniques to study the distribution of myelin and glial constituents in experimental and human demyelinating diseases. Several projects are concerned with the role of myelin-associated glycoprotein (MAG is localized in periaxonal regions of myelin-forming oligodendroglia) in myelin sheath formation, maintenance and breakdown. In the mouse mutant, jimpy, oligodendroglia are decreased in number, produce less MAG and basic protein (BP is a major protein constituent of compact myelin) than controls and form very few myelin sheaths. Since less MAG and BP are present in oligodendroglial perikarya and processes, synthesis and transport of these constituents are abnormal. Since they also accumulate periaxially, insertion of myelin components into developing lamellae also appears to be defective. Experimental allergic encephalomyelitis (EAE) has been used as a model for multiple sclerosis and is characterized by perivenous demyelination and relative preservation of axons. As demyelinating lesions develop in rats, changes in MAG and BP are similar in distribution and are found in regions where myelin sheath abnormalities can be identified in the electron microscope. Even though many features of EAE lesions vary with the differences in species used, regions of CNS examined, or sensitizing agents injected, it is of interest that the differences in MAG and BP staining pattern we identified in multiple sclerosis are not found in this type of EAE. An immunocytochemical study of hexachlorophene (HCP) intoxication provides additional evidence that MAG is localized periaxially in myelinated fibers. HCP intoxication was studied because it produces severe myelin sheath vacuolation that does not progress to demyelination. Instead, the vacuoles decrease in size and number if the drug is stopped and recovery occurs. Even when sheaths are severely vacuolated, they stain normally with BP antiserum. MAG staining is limited to oligodendroglial cytoplasm around myelinated axons. As blisters increase in size, it does not spread to myelin lamellae or vacuolar spaces; the normal MAG staining pattern persists also during recovery.

Two projects are concerned with the distribution of myelin and glial constituents in human demyelinating diseases. In the first, multiple sclerosis lesions are being examined for evidence of myelin regeneration by oligodendroglia (during CNS myelin formation, oligodendroglia are intensely stained by BP antiserum) and by Schwann cells (PNS myelin formed in the CNS by Schwann cells can be detected by immunostaining with antiserum to P_0 , a major protein constituent of PNS myelin). The results show that in spinal cord lesions of MS, Schwann cells may form PNS myelin around CNS axons during the active phase of CNS demyelination. Later, in "shadow" plaque areas where some

myelinated axons persist, oligodendrocytes that are stained by BP antiserum extend processes to demyelinated axons and remyelinate them. In the second project, lesions of progressive multifocal leukoencephalopathy are being immunostained with antisera to JC virus, MAG, and BP. In sections from a rapidly progressive case, JC virus antiserum stained occasional single oligodendroglia in white matter that appears normal histologically. In zones surrounding areas of demyelination, virus-containing oligodendroglia are most numerous, MAG staining of periaxonal regions is decreased, but there is little change in BP staining of myelin. In more chronic cases, viral antiserum stains fewer oligodendrocytes and the differences in MAG and BP staining are much less striking. These observations suggest that altered periaxonal MAG staining is an early sign of oligodendroglial disease and it precedes the breakdown of myelin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02322-03 LNNS																
PERIOD COVERED October 1, 1979 to September 30, 1980																		
TITLE OF PROJECT (80 characters or less) Permeability of the blood-brain barrier (BBB) to norepinephrine (NE) in experimental cerebral ischemia																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">H. Hervonen</td> <td style="width: 30%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>O. Steinwall</td> <td>Visiting Scientist</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	H. Hervonen	Visiting Fellow	LNNS NINCDS	Other:	O. Steinwall	Visiting Scientist	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS															
COOPERATING UNITS (if any) None																		
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SECTION Section on Cerebrovascular Pathology																		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: <div style="text-align: center;">0.95</div>	PROFESSIONAL: <div style="text-align: center;">0.85</div>	OTHER: <div style="text-align: center;">0.1</div>																
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS											
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<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS																	
SUMMARY OF WORK (200 words or less - underline keywords) This project has been completed. Part of the results were presented at the Symposium on Cerebral Microvasculature: Investigation of the Blood Brain Barrier, Galveston, July 1979. <div style="margin-left: 40px;"> Hervonen, H., Steinwall, O., Spatz, M., and Klatzo, I.: Behaviour of the blood-brain barrier toward biogenic amines in experimental cerebral ischemia. In: <u>Advances in Experimental Medicine and Biology</u>, Vol. 131. <u>The Cerebral Microvasculature</u>. New York, Plenum Press, 1980, pp. 295-306. </div>																		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02356-02 LNNS														
PERIOD COVERED October 1, 1979 to September 30, 1980																
TITLE OF PROJECT (80 characters or less) Studies on resolution of the vasogenic brain edema (VBE)																
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	K. Fujiwara	Visiting Fellow	LNNS NINCDS													
	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS													
COOPERATING UNITS (if any) None																
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SECTION Section on Cerebrovascular Pathology																
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																
TOTAL MANYEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">1.0</div>	OTHER: <div style="text-align: center;">0.1</div>														
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SUMMARY OF WORK (200 words or less - underline keywords) <p> Mechanisms of resolution of the vasogenic brain edema (VBE) were studied by application of <u>specific gravity</u> measurements correlated with <u>immunocytochemical</u> observations on <u>extravasated serum proteins</u>. The main mechanism of VBE resolution appears to be related to <u>intracellular uptake of serum proteins by the glial cells</u>. This project has been completed. </p>																

Project Description:

Objectives: The main objective of this study is to elucidate the mechanisms which are responsible for the resolution of the VBE. This could lead to designing effective therapeutic measures for the treatment of brain edema patients.

Methods Employed: Cortical cold injury in cats served as the model of VBE. As a marker for the spreading of edema fluid, Evans Blue dye was injected before the operation to produce the cold injury. The animals were sacrificed after various time intervals. The brains were sectioned into two coronal blocks, one of which was immediately submerged into kerosene and the other subjected to paraformaldehyde fixation. The block in kerosene was photographed with a Color Polaroid camera and the samples to be taken for specific gravity measurements were marked on the photograph. The specific gravity of the samples from areas of edema and control regions was measured in gradient columns and the values were marked on another color photograph of the same block. The coronal block of the brain fixed in paraformaldehyde was subjected to immunocytochemical procedures to demonstrate the localization of serum proteins in vibratome-cut sections.

Major Findings: The plotting of specific gravity measurements on the coronal sections of brains with cold lesions visualized progression and resolution of brain edema at various time intervals following cold injury. The edema was spreading preferentially through the white matter into the gyri adjacent to the injury. The resolution of edema was observed to take place from the periphery toward the site of the lesion. This coincided with the dramatic uptake of extravasated serum proteins in the extracellular spaces by the glial cells, and particularly by the astrocytes.

Significance to Biomedical Research and the Program of the Institute: The described findings allow proposal of the following hypothesis for the mechanism of resolution of VBE. The enhanced content of water in edematous areas is related to the presence of extracellular, extravasated serum proteins. The resulting shift in normal difference (25 mm Hg) between the colloidal osmotic pressure of the plasma and the interstitial fluid is responsible for retention of water in the edematous white matter. The vigorous intragial uptake of serum proteins reinstates the basic normal relationship of transcapillary flow according to Starling's law restoring differences in hydrostatic and osmotic colloidal pressures. The water unbound from the proteins diffuses away and this constitutes the main mechanism for the resolution of the VBE.

Proposed Course of the Project: This project has been completed. The findings were presented at the Int. Symposium on Brain Edema, Sept. 1979, Berlin.

Publications:

Klatzo, I., Chui, E., Fujiwara, K., and Spatz, M.: Resolution of vasogenic brain edema (VBE). In Cervós-Navarro, J., and Ferszt, R. (Eds.): Advances in Neurology, Vol. 28, Brain Edema. New York, Raven Press, 1980, pp. 359-374.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02425-01 LNNS														
PERIOD COVERED October 1, 1979 to September 30, 1980																
TITLE OF PROJECT (80 characters or less) Glial Fibrillary acidic protein (GFAP) reaction in astrocytes																
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TOTAL MANYEARS: <div style="text-align: center;">1.30</div>	PROFESSIONAL: <div style="text-align: center;">1.20</div>	OTHER: <div style="text-align: center;">0.1</div>														
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SUMMARY OF WORK (200 words or less - underline keywords) <p> Using peroxidase-antiperoxidase (PAP) procedure designed to demonstrate the <u>GFA</u> <u>protein</u> by specific antibody the basic astrocytic reaction consisting in <u>pro-</u> <u>liferation</u> of astroglial fibrils was studied in cats subjected to the <u>occlusion</u> <u>of the middle cerebral artery (MCA)</u> or to <u>cerebral embolism</u> by injecting solid <u>microspheres</u> into the internal carotid artery. These studies revealed that the GFAP reaction appearing as a striking proliferation of fibrils specifically confined to astrocytes was demonstrable in cats sacrificed only 10 minutes after embolization. In cats with MCA occlusion and sacrificed after 6 hours extensive GFAP reaction was conspicuous in the regions of the brain affected by cerebral ischemia with the exception of the areas which showed extravasation of serum proteins. </p>																

Project Description:

Objectives: The main objective of this study is to elucidate the mechanism of the most basic and common pathological reaction in the brain tissue, namely, the astrocytic gliosis.

Methods Employed: The immunocytochemical procedure of peroxidase-antiperoxidase (PAP) was applied to demonstrate the glial fibrillary acidic protein (GFAP) by specific antibody in cats subjected to cerebral ischemia by MCA occlusion or to cerebral embolism by injecting solid microspheres into the internal carotid artery. The cats were injected with the 2% Evans Blue (EB) and sacrificed at different time intervals after an ischemic insult. The ischemic insult was ascertained also by specific gravity measurements of 2 mm in diameter brain tissue samples excised from the areas of interest.

Major Findings: The cats subjected to microembolization showed a very marked proliferation and hypertrophy of astrocytic fibrils which reacted strongly with GFAP antibody in the PAP method. This striking astroglial reaction was present also in the animals sacrificed at the shortest time interval, i.e., after 10 minutes. In the normal brain regions, not affected by the emboli, the astrocytes contained only very few, weakly stained fibrils. In cats subjected to MCA occlusion and sacrificed at 6 hrs interval the GFAP reaction was observed in the areas affected by ischemia by showing no exudation of EB tracer or the PAP staining for serum proteins. These areas revealed also lower specific gravity values indicating the presence of ischemic cytotoxic edema.

Significance to Biomedical Research and the Program of the Institute: This study represents an effort to unravel the mechanisms responsible for the most basic pathological reaction in the brain tissue, i.e., astrocytic gliosis. Our findings indicate that formation of GFAP can be stimulated very quickly (i.e., within 10 min) and it is not related to the exudation of serum proteins but to the ischemic effect on the tissue. These observations favor a possibility that GFAP reaction takes place due to polymerization of GFA protein present in the astrocytic cytoplasm in the liquid form. This may occur due to changes in permeability of astrocytic cell membranes and an abnormal entry of some ions, such as calcium or magnesium.

Proposed Course of the Project: This project will be continued to elucidate further the circumstances under which the GFAP reaction takes place.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02426-01 LNNS																
PERIOD COVERED October 1, 1979 to September 30, 1980																		
TITLE OF PROJECT (80 characters or less) Studies on cerebral embolism produced by injection of microspheres into the internal carotid artery in cats																		
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SECTION Section on Cerebrovascular Pathology																		
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TOTAL MANYEARS: <div style="text-align: right; margin-right: 20px;">2.35</div>	PROFESSIONAL: <div style="text-align: right; margin-right: 20px;">2.25</div>	OTHER: <div style="text-align: right; margin-right: 20px;">0.1</div>																
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SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Ischemic effect of microembolization</u> was studied in cats correlating several parameters of injury. In the same or directly adjacent brain sections the permeability of the blood-brain barrier (BBB) to proteins and water content of the tissue were compared. <u>Immunocytochemical (PAP) methods</u> revealed a close correlation between the extravasated serum proteins and the edematous changes evaluated by the specific gravity measurements. Immunocytochemical staining for the glial fibrillary acidic protein (GFAP) demonstrated a prompt fibrillary reaction of the astrocytes occurring as early as 10 minutes after injection of microspheres. </p>																		

Project Description:

Objectives: This study was designed to evaluate further the inter-relationship between an injury of the BBB resulting in exudation of serum proteins and the dynamics of associated brain edema. The other purpose was to study the effect of BBB breakdown on the astrocytic glial fibrils.

Methods Employed: Under Nembutal anesthesia the cats were injected with carbonized microspheres ($15 \pm 5 \mu$ in diameter) into the carotid circulation through a catheter introduced into the left lingual artery. Shortly preceding the microembolization the cats received 2 ml/Kg of 2% Evans Blue (EB) solution. Groups of experimental animals were sacrificed at different time intervals, ranging from 10 minutes to 4 weeks following the administration of microspheres. Coronal blocks of the brains were either placed immediately into the kerosene for specific gravity measurements or submerged into the 10% buffered paraformaldehyde for the PAP methods. Frozen, paraformaldehyde fixed sections were observed for distribution of the EB under the fluorescence microscope. In half of the experimental animals the brains were fixed by glutaraldehyde and paraformaldehyde for electron microscopic and immunocytochemical studies.

Major Findings: The cats injected with microspheres and sacrificed 10 minutes later showed extensive leakage of serum proteins into the brain tissue. The PAP method revealed at that time a "flea-bitten" pattern with extravasations located primarily in the gray matter. The PAP procedure for GFAP revealed in the areas related topographically to embolic foci astrocytes displaying dense bundles of fibrils. In animals sacrificed later than one day the exudation of serum proteins shifted towards white matter. After several days and later there was evidence of disappearance of extravasated serum proteins and this coincided with the resolution of edematous changes. The strong glial fibrillary reaction persisted in all animals.

Significance to Biomedical Research and the Program of the Institute: These studies on effect of microemboli represent another approach to the study of various mechanisms involved in cerebral ischemia. The present observations revealed differences in the behavior of the BBB, which is almost instantaneously broken in cerebral embolization, whereas it becomes permeable to serum proteins only after several hours following occlusion of a major cerebral artery. These studies provide further support to the hypothesis that resolution of brain edema is related to the glial uptake of extravasated, extracellular serum proteins.

Proposed Course of the Project: These studies will be supplemented by observations on the duration of the BBB opening and its permeability to other than protein tracers.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02427-01 LNNS									
PERIOD COVERED October 1, 1979 to September 30, 1980											
TITLE OF PROJECT (80 characters or less) Observations on changes in cerebrovascular permeability to proteins in the stroke-prone, spontaneously hypertensive rats (SPRSH)											
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M. A. Greenwood	Biologist	LNNS NINCDS									
COOPERATING UNITS (if any) None											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Cerebrovascular Pathology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: <div style="text-align: center;">1.2</div>	PROFESSIONAL: <div style="text-align: center;">0.6</div>	OTHER: <div style="text-align: center;">0.6</div>									
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SUMMARY OF WORK (200 words or less - underline keywords) <p>The <u>permeability of cerebral blood vessels to proteins</u> was studied in the stroke-prone, spontaneously hypertensive rats (SPRSH) by immunocytochemical method (PAP) designed for demonstration of extravasated serum proteins. As controls served the normotensive Wistar-Kyoto rats and spontaneously hypertensive but stroke-resistant animals (SHRSR). In SPRSH, even in the presymptomatic animals, application of peroxidase-antiperoxidase (PAP) method to demonstrate serum proteins revealed very extensive areas of protein extravasations in the white matter and a striking localization of serum proteins in the cytoplasm of the adjacent neurons. The testing of the blood-brain barrier (BBB) with conventional tracers revealed no or only minimal changes of the BBB. This project is completed.</p>											

Project Description:

Objectives: The main objective of this project is to evaluate the changes in cerebrovascular permeability to serum proteins in a chronic hypertension using the strain of spontaneously hypertensive rats as the experimental model.

Methods Employed: The following groups of animals were used in this investigation: (I) SPRSH (26 animals) with blood pressure ranging 210-230 mmHg, (II) SHRSR (10 animals) in which the blood pressure was between 150-200 mmHg, and (III) normal Kyoto-Wistar rats (10 animals) with the blood pressure ranging 100-150 mmHg. 2% Evans Blue 0.2 ml/100 g b.w. was injected intravenously one hour before the sacrifice. The rats were sacrificed by perfusion with 10% paraformaldehyde at approximately 9 months of age. The coronal blocks of the brain were sectioned on the vibratome and processed according to the PAP method to demonstrate the distribution of rat serum proteins. Some blocks were embedded in paraffin and the sections were stained with H & E and Nissl methods.

Major Findings: The control groups of stroke-resistant hypertensive and Kyoto-Wistar rats showed no changes. In the SPRSH group 9 animals with CNS symptoms showed histopathological changes characteristic of hypertensive encephalopathy (cystic defects, hemorrhagic foci, softenings etc.). The PAP methods showed extensive areas of serum protein extravasation especially conspicuous in the white matter. Microscopically the serum proteins in the white matter were located either extracellularly or were taken up by glial cells. In the adjacent lower layers of the cortex the serum proteins were conspicuous in the cytoplasm of the neurons. Numerous small hemorrhagic foci could be observed without any evidence of protein extravasation. Similar findings with the PAP method were observed also in several presymptomatic rats in which the H & E and Nissl stains revealed no appreciable histopathological changes. The BBB testing with Evans Blue tracer showed in these animals no or only very small and few foci of tracer extravasation.

Significance to Biomedical Research and the Program of the Institute: The importance of these studies lies in the fact that they are undertaken in an animal model which resembles the human hypertensive encephalopathy. The most significant finding of this study relates to the observation that the presymptomatic rats showed extensive chronic leakage of serum proteins which was unexpected and surprising in view of the lack of any clinical symptoms and of any appreciable histopathological changes in the brain.

Proposed Course of the Project: This project is almost completed. Some additional observations will be made using PAP staining for the glial fibrillary proteins.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01999-08 LNNS
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Transport studies in ischemic cerebral edema		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: M. Spatz Head, Section on Neurocytobiology LNNS NINCDS Other: I. Klatzo Chief, Lab. Neuropath. Neuroanat. Sci. LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytobiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been temporarily discontinued.		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02000-08 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Biochemistry of brain edema in cerebral ischemia of gerbils Former title: Brain edema in cerebral ischemia of gerbils														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">M. Spatz</td> <td style="width: 40%;">Head, Section on Neurocytobiology</td> <td style="width: 20%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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Other:	K. Abe	Visiting Fellow	LNNS NINCDS											
	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS											
COOPERATING UNITS (if any) B. B. Mrsulja, Institute of Biochemistry, Faculty of Medicine, Belgrade, Yugoslavia														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.2</div>												
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SUMMARY OF WORK (200 words or less - underline keywords) <u>Biochemical parameters</u> of various cerebral metabolic pathways were <u>correlated</u> with the <u>changes in water content</u> of gerbils' brains subjected to 15 minutes of bilateral <u>cerebral ischemia</u> with and without recovery. The findings suggested that Na-K-ATPase, serotonin and cyclic AMP are some of the most important factors involved in the development and/or persistence of ischemic cerebral edema.														

Project Description:

Objectives: In human cerebral ischemia, brain edema is considered to be an important factor in causing mortality (Shaw, C., Alvord, E., and Berry, R., Arch. Neurol. 1: 161-177, 1959). Experimentally, cerebral ischemia can be easily produced in Mongolian gerbils by ligation of a single common carotid artery (Levine, S., Payan, H., Exp. Neurol. 16: 252-255, 1966; Kahn, K., Arch. Pathol. 69: 544-553, 1972; Ito et al., Acta Neuropath. 32: 209-223, (1975)). The experimental evidence suggests that brain edema in focal ischemia begins as a cytotoxic type and is followed by a vasogenic type. Recently we have shown that the release of arterial occlusion and the reestablishment of the circulation in the gerbil brain markedly accelerate the formation of edema. To elucidate the mechanism responsible for these observations, an attempt was made to define the biochemistry of the postischemic edema and correlate it with the water content of the brain.

Methods Employed: Several groups of adult gerbils were subjected to 15 minutes of bilateral carotid artery occlusion and various periods of recovery. The following biochemical parameters were determined in experimental and sham-operated animals: a) adenylate nucleotides (AMP, ADP, ATP, cyclic AMP) and lactate in cerebral cortex; b) biogenic amines (DA, NE and 5-HT) in cerebral cortex and caudate; c) enzymes of glycolysis, glycogen metabolism, pentose monophosphate pathway, GABA-metabolizing enzymes, some key enzymes in Krebs cycle, Na-K-ATPase in the capillaries and parenchyma of the brain; d) the estimation of water accumulation was determined by measuring the specific gravity of the brain structures.

Major Findings: Brain metabolites closely related to energy production are found changed in ischemia but are quickly restored after recirculation although ischemic brain edema develops. At the same time, significant changes in the levels of biogenic amines (5-HT, DA and NE) are found in the brain. Moreover, the capillary but not the parenchymal Na-K-ATPase activity was reduced in ischemia. Correlations of ischemic brain edema with the measured biochemical parameters were indicative of the following processes: a) enzymes of the main energy-producing systems are activated with the increase of water content; b) decrease in the activities of the ATPase system (particularly Na-K-ATPase) increases the water content; c) biogenic amines, the GABA system and the rest of the measured enzymatic activities, as well as high-energy phosphate metabolites, show no linear correlation with the development or persistence of postischemic brain edema. However, the changes in serotonin are mirror images of the water content, shifted in time toward the onset of recirculation. Thus, many factors, as well as their possible interreactions, are involved in the development and/or persistence of ischemic brain edema. Among them, factors of the utmost importance are Na-K-ATPase, serotonin and cyclic AMP.

Part of this study was presented in Berlin at the International Symposium on Brain Edema - Pathology and Therapy, September 1979.

Significance to Biomedical Research and the Program of the Institute: Cerebral edema occurs as one of the major complications of many neurological disorders such as ischemia, trauma, tumors, chemical poisoning, and others. The basic understanding of the type of edema and its development is very crucial for the clinician who is faced not only with the diagnosis, but with the appropriate selection of treatment. Thus, various investigations of this problem are essential for finding the factor or factors responsible for the occurrence of cerebral edema and its treatment.

Proposed Course of the Project: The effect of preischemic and post-ischemic treatment will be investigated in the various biochemical events occurring in ischemic cerebral edema.

Publications:

Mrsulja, B. B., Djuricić, B. M., Cvejić, V., Mrsulja, B. J., Abe, K., Spatz, M., and Klatzo, I.: Biochemistry of experimental ischemic brain edema. In Cervós-Navarro, J., and Ferstz, R. (Eds.): Advances in Neurology, Vol. 28. Brain Edema. New York, Raven Press, 1980, pp. 217-230.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02001-08 LNNS						
PERIOD COVERED October 1, 1979 to September 30, 1980								
TITLE OF PROJECT (80 characters or less) Amino Acids Transport in Hypoxia, Hypercapnia and Hypocapnia								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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COOPERATING UNITS (if any) None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0						
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SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated.								

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PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Properties of cerebral capillaries in organotypic cultures														
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LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
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SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated.														

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02165-06 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Correlation of ³ H isoleucine uptake in pia arachnoid with culture of fibroblasts														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">M. Spatz</td> <td style="width: 40%;">Head, Section on Neurocytobiology</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other:	M. R. Murray	Research Biologist	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS											
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COOPERATING UNITS (if any) None														
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TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>												
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SUMMARY OF WORK (200 words or less - underline keywords) This project has been temporarily discontinued.														

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02197-05 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Demonstration of ATPase in cerebellar cultures														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">M. Spatz</td> <td style="width: 40%;">Head, Section on Neurocytobiology</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other:	M. R. Murray	Research Biologist	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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COOPERATING UNITS (if any) None														
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PERIOD COVERED October 1, 1979 to September 30, 1980								
TITLE OF PROJECT (80 characters or less) Ischemic and postischemic effect on the uptake of neutral amino acids in isolated cerebral capillaries								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Cerebral capillary endothelial cultures														
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SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.2</div>												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS							
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER												
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS													
SUMMARY OF WORK (200 words or less - underline keywords) The established cerebral endothelial cell cultures are capable of taking up L-dopa but no other monoamines and do not show pinocytotic activity in the presence of horseradish peroxidase as the tracer. Hence, they provide an ideal model for the studies on the mechanisms involved in the induction of pinocytosis and/or vesicular transport in pathological conditions.														

Project Description:

Objectives: The continuous study of the established cerebral endothelial cultures has been concerned with investigating their capability of monoamine uptake and pinocytotic activity which are known to be limited in the normal cerebral capillaries.

Methods Employed: Washed and preincubated cultures were incubated with L-dopa, dopamine, norepinephrine, alpha-methyl-norepinephrine, epinephrine or serotonin (concentration 10^{-3} - 10^{-5} M) with or without inhibition of monoamine oxidase (Pargyline 10^{-3} M) at 36°C for 10 minutes. Para-chloromercuribenzenesulfonic acid (10^{-4} M) was used as amino acid transport inhibitor in the L-dopa uptake studies. Cultures incubated without the substrates served as controls. The monoamines' fluorescence was demonstrated histochemically using the glyoxylic acid method (Björklund et al., 1972). For the demonstration of pinocytotic activity, the prewashed cultures were preincubated with glucose (600 mg %) and bovine albumin (10 mg/ml) for 10 minutes, then reincubated with 10 mg/ml of horseradish peroxidase (HRP) replacing the albumin for 10 minutes. Thereafter the cultures were processed for histochemical demonstration of HRP by light and electron microscopy.

Major Findings: The endothelial cell aggregates showed histofluorometrically demonstrable L-dopa uptake which was reduced in the presence of p-chloromercuribenzenesulfonic acid. However, none of the tested monoamines were taken up by these cells irrespective of whether they were incubated with or without the MAO inhibitor Pargyline. Furthermore, pinocytosis was not seen using horseradish peroxidase tracer. Thus, the endothelial cells in these cultures acted in respect to the monoamines, and L-dopa uptake, as well as to their pinocytotic activity, identically as those in the brain microvessels in vivo.

Significance for Biomedical Research and the Program of the Institute: The established cerebral capillary endothelial cell cultures provide a pure cell line which will be useful for the investigation of cerebral endothelial cells in the living state without the influence of any other cells. Thus, the function of cerebral capillary endothelium as compared to endothelium derived from capillaries outside the blood-brain barrier (BBB) system can be characterized under normal and pathologic conditions. This approach will also add another dimension for the studies related to the BBB permeability in various disease processes.

Proposed Course of the Project: The primary objective of this project has been to obtain an easily reproducible endothelial cell line to elucidate the mechanism involved in their unique function as the constituents of the BBB. Another manuscript is in preparation besides the already published method for cultivation of cerebral capillary endothelium.

Publications:

Spatz, M., Bembry, J., Dodson, R. F., Hervonen, H., and Murray, M. R.:
Endothelial cell cultures derived from isolated cerebral microvessels.
Brain Res. 191: 577-582, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02280-04 LNNS
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) The effect of cerebral ischemia and postischemia on monoamine oxidase (MAO) activity		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: M. Spatz Head, Section on Neurocytobiology LNNS NINCDS Other: W. D. Rausch Visiting Fellow LNNS NINCDS K. Abe Visiting Fellow LNNS NINCDS		
COOPERATING UNITS (if any) D. Mičić, Institute of Biochemistry, Faculty of Medicine, Belgrade, Yugoslavia		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytobiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.9	PROFESSIONAL: 0.8	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been completed and the resulting manuscript is in press. Mičić, D. Abe, K., Rausch, W. D., and Spatz, M.: The ischemic and post-ischemic effect on the activities of cerebral monoamine oxidase, cytochrome oxidase and acetylcholinesterase in Mongolian gerbils. In Spatz, M., Mrsulja, B. B., Rakić, Lj. M., and Lust, W. D. (Eds.): <u>Circulatory and Developmental Aspects of Brain Metabolism</u> . New York, Plenum Press, 1980, pp. 81-96.		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02324-03 LNNS																
PERIOD COVERED October 1, 1979 to September 30, 1980																		
TITLE OF PROJECT (80 characters or less) Studies on the blood-brain barrier (BBB) to 5-hydroxytryptamine Former title: The ³ H norepinephrine uptake and fate in the isolated cerebral capillaries																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">T. Abe</td> <td style="width: 40%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>W. D. Rausch</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	T. Abe	Visiting Fellow	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		W. D. Rausch	Visiting Fellow	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
PI:	T. Abe	Visiting Fellow	LNNS NINCDS															
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	W. D. Rausch	Visiting Fellow	LNNS NINCDS															
	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS															
COOPERATING UNITS (if any) None																		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																		
SECTION Section on Neurocytobiology																		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: <div style="text-align: center;">1.0</div>	PROFESSIONAL: <div style="text-align: center;">0.9</div>	OTHER: <div style="text-align: center;">0.1</div>																
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>																		
SUMMARY OF WORK (200 words or less - underline keywords) The uptake of 5-hydroxytryptamine (5-HT) takes place by a specific Na ⁺ and K ⁺ dependent carrier-mediated process and it is metabolized in the isolated cerebral microvessels. 5-Hydroxyindole-3-acetic acid (5-HIAA) was found to be the main metabolite extractable from the microvessels and the incubating media. Anoxia markedly reduced the metabolic rate of capillary 5-HT.																		

Project Description:

Objectives: Only a slight amount of circulating 5-hydroxytryptamine (5-HT) was found to pass the blood-brain barrier (Axelrod and Inscoe, J. Pharmacol. Exp. Therap. 141: 161, 1963). To elucidate the mechanism responsible for these reported observations the uptake and metabolism of radiolabeled 5-HT were investigated in isolated cerebral microvessels, which were previously proven to take up and metabolize norepinephrine.

Methods Employed: The isolated cerebral capillaries were incubated with ^3H or ^{14}C 5-HT in Ringer's solution containing .1% albumin (pH 7.4) alone or with various concentrations of unlabeled (cold) 5-HT, norepinephrine, L-dopa, dopamine, epinephrine, metaraminol, normetanephrine and metanephrine for various periods of time. The effect of various amino acids, metabolic inhibitors, adrenergic blocking agents, hypothermia and anoxia on the capillary uptake of 5-HT was determined under appropriate respectively changed conditions of the incubating medium.

Major Findings: The capillary uptake of ^3H 5-HT was found to be saturable since it could be inhibited by increasing the concentration of unlabeled (cold) 5-HT in the incubating medium containing the labeled substrate (estimated K_m 2.3 μM). The addition of $^3\text{K}^+$ increased while the reduction of Na^+ or addition of ouabain decreased the ^3H 5-HT uptake in the isolated microvessels. Metabolic inhibitors (Na azide, NaFl, DPN, KCN) had little effect on either the uptake or metabolism of labeled 5-HT in the microvessels. However, hypothermia, phentolamine (1 mM), dichloroisoproterenol (1 mM) and imipramine (0.3 mM) reduced the capillary 5-HT uptake in 90, 86, 83 and 57 percent, respectively. Moreover, a cross inhibition of this uptake was seen by L-norepinephrine, DL metaraminol, L-epinephrine, L-dopa, dopamine and tryptamine but not by the amino acids of the L and A transport system. Bivalent ions (Co^{++} Mn^{++}) reduced both the uptake and metabolism of ^{14}C 5-HT. 5-HIAA was found to be the main metabolite extractable from the microvessels and from the incubating medium. The turnover rate of ^{14}C 5-HT was 1.89 nmoles/mg P/30 minutes. Anoxia markedly reduced the metabolic rate of the labeled 5-HT.

Significance to Biomedical Research and the Program of the Institute: These results indicate that the capillary uptake of 5-HT takes place by a specific Na^+ and K^+ dependent carrier-mediated process (which may be shared by other amines). These features and the sensitivity of the 5-HT uptake to hypothermia and to metaraminol cross inhibition resemble the reported characteristic properties of the neuronal amines' uptake. However, the cerebral microvessels also showed the main characteristics of extraneuronal uptake, namely, the capability of metabolizing the 5-HT and releasing the deaminated metabolite. Thus, the cerebral microvessels have the capacity for 5-HT uptake and metabolism rather than for uptake and storage. Therefore, they are

most likely responsible for regulating the inflow and outflow of 5-HT by inactivating the amine, which can be altered under pathological conditions.

Proposed Course of the Project: These investigations have been extended to other members of the catecholamine family. Part of the work was presented at the Symposium on Cerebral Microvasculature: Investigation of the Blood Brain Barrier, in Galveston, July 1979, and part will be presented at the Neuroscience meeting in 1980.

Publications:

Abe, T., Abe, K., Rausch, W. D., Klatzo, I., and Spatz, M.: Characteristics of some monoamine uptake systems in isolated cerebral capillaries. In: Advances in Experimental Medicine and Biology, Vol. 131. The Cerebral Microvasculature. New York, Plenum Press, 1980, pp. 45-55.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02327-03 LNNS									
PERIOD COVERED October 1, 1979 to September 30, 1980											
TITLE OF PROJECT (80 characters or less) The study of monoamines' uptake and pinocytotic activity of pia arachnoid cultures. Former title: The uptake of biogenic amines into the cells of pia arachnoid cultures											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: H. Hervonen</td> <td style="width: 33%;">Visiting Fellow</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> <tr> <td>M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> </table>			PI: H. Hervonen	Visiting Fellow	LNNS NINCDS	Other: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	M. R. Murray	Research Biologist	LNNS NINCDS
PI: H. Hervonen	Visiting Fellow	LNNS NINCDS									
Other: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS									
M. R. Murray	Research Biologist	LNNS NINCDS									
COOPERATING UNITS (if any) None											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Neurocytobiology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.6	OTHER: 0.2									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS				
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<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords) The <u>pia arachnoid membrane</u> as a constituent of the blood-brain barrier (BBB) has been cultivated and evaluated in regard to the <u>monoamine uptake</u> and <u>pinocytotic activity</u> which are restricted by the BBB <u>in vivo</u> . Both cell types (endothelium and pia) of the pia arachnoid membrane showed a limited uptake of catecholamines and serotonin. L-dopa was taken up by the endothelial but not by the pial cells while the reverse situation was observed in regard to the pinocytotic activity.											

Project Description:

Objectives: Pia arachnoid and its blood vessels constitute one of the sites where the brain and cerebrospinal fluid (CSF) are separated from the blood and external tissue by so-called blood-brain barrier (BBB) (Rapoport, S., Blood-brain Barrier in Physiology and Medicine, Raven Press, N.Y., 1976).

The aim of this study has been to evaluate the characteristics of pia arachnoid cellular components toward biogenic amines and pinocytotic activity which are restricted by the BBB in vivo.

Methods Employed: The pia arachnoid membrane was prepared from newborn rats and cultured on glass in a Maximow double coverslip assembly for 2-3 weeks.

The incubations for the catecholamine uptake were performed in a HEPES-buffered (20 mM) Locke's salt solution, pH 7.4, at room temperature. The cultures were first briefly washed to remove the culture medium, then preincubated for 10 minutes with or without pargyline (a monoamine oxidase inhibitor) and pyrogallol (a catechol-O-methyl transferase inhibitor). The incubation time was 10 minutes, again with or without pargyline and pyrogallol according to the preincubation. The following biogenic amines and precursors were used in 10^{-5} to 10^{-2} M concentrations: L-dopa, dopamine, noradrenaline, adrenaline and serotonin. After incubation the cultures were washed in HEPES-LOCKE's solution for 5 seconds to 10 minutes before processing for either formaldehyde-induced fluorescence or glyoxylic acid-induced fluorescence.

Zeiss Axiomat microscope was used to observe the fluorescence operating either with transmitted light with EG 12 excitation filter, dark-field condensor and LP 500 barrier filter or with epi-illumination with BG 12 and BP 405 excitation filters, LP 470 barrier filter and a dichroic mirror. The same microscope was used for phase-contrast microscopy. Unstained or stained (uranyl acetate and lead citrate) thin sections were examined with Philips EM 300 and JEOL 100C electron microscopes.

Major Findings: Two populations of cells have been identified in the cultures of the pia arachnoid membrane by both light and electron microscopy: 1) endothelial cells and (2) pia arachnoid cells. Specific L-dopa uptake and accumulation of biogenic amines were demonstrated with glyoxylic acid histochemistry in the endothelial cells but not in the pia arachnoid cells. Uptake of the monoamines was of extraneuronal type (Iversen, L.L., Brit. J. Pharmacol. Chemotherap. 25, 18-33, 1965) and was found to be equally limited in both cell types. The pia arachnoid cells show a high pinocytotic activity by removing the horseradish peroxidase from the incubating medium. However, the endothelial cells did not display any signs of pinocytosis or vesicular transport showing virtually no intracellular horseradish peroxidase.

Significance to Biomedical Research and the Program of the Institute:
The pia arachnoid offers a relatively simple in vitro model for the study of factors influencing pinocytosis and the vesicular transport in the cellular constituents of the BBB which are altered in many pathological conditions.

Proposed Course of the Project: This work is completed and the manuscript is being prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02328-03 LNNS						
PERIOD COVERED October 1, 1979 to September 30, 1980								
TITLE OF PROJECT (80 characters or less) The effect of cholinesterase inhibitors on nerve cells developing in cultures of spinal and sympathetic ganglia								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: H. Hervonen</td> <td style="width: 33%;">Visiting Fellow</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> </table>			PI: H. Hervonen	Visiting Fellow	LNNS NINCDS	Other: M. R. Murray	Research Biologist	LNNS NINCDS
PI: H. Hervonen	Visiting Fellow	LNNS NINCDS						
Other: M. R. Murray	Research Biologist	LNNS NINCDS						
COOPERATING UNITS (if any) None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.2</div>						
CHECK APPROPRIATE BOX(ES) <table style="width: 100%;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS	
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER						
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS							
SUMMARY OF WORK (200 words or less - underline keywords) The inhibition of cholinesterases, especially <u>acetylcholinesterase</u> , in the <u>developing neuroblast of the spinal and sympathetic ganglion</u> leads to a growth inhibition and degeneration of the neurons, strongly supporting the hypothesis that <u>acetylcholinesterase</u> plays an important role in the <u>maturation of the neurons</u> .								

Project Description:

Objectives: It is known that acetylcholinesterase (AChE) appears early in developing neurons before the onset of cholinergic transmission and it was thought that the sensory ganglia might provide some crucial information in this matter since these are neither cholinergic nor cholinceptive. The purpose of this work was to inquire into the possible role of cholinesterase in the general maturation of neurons aside from their accepted enzymatic functions in neurotransmission.

Methods Employed: The spinal and sympathetic ganglia were prepared from 8-day-old chick embryos and cultured in Maximow double coverslip assembly up to 4 weeks in vitro. The inhibitors were added to the culture medium for the whole culture period in concentrations 10^{-6} to 10^{-3} M. The following inhibitors were used: Eserine (physostigmine), iso-OMPA, BW 274 C 51, DFP and paraoxon. Observations in the perikaryal morphology and size (growth and differentiation) as well as fiber outgrowth were made on the living and stained ganglia at successive stages in their development.

Major Findings: At concentrations of 10^{-3} M iso-OMPA affects the neuron somewhat unfavorably but does not destroy as do the other substances which inhibit AChE. From this it appears that AChE performs some function in neuron development which is not related to neurotransmission. These results were reported by invitation at the meeting of the Peripheral Nerve Study Group at Wye College, England, July 11-14, 1979: "Effects of cholinesterase inhibition on developing peripheral ganglion cells in culture" (Murray, M.R., and Hervonen, H.). In all but the nonspecific inhibitor (iso-OMPA) of AChE, unfavorable changes were observed in the cultures even at the concentrations as low as 10^{-7} M.

Significance to Biomedical Research and the Program of the Institute: The significance of this study is to further explore the role(s) of an enzyme/a group of enzymes (acetylcholinesterase/cholinesterases) which have a widespread occurrence in the nervous system.

Proposed Course of the Project: This investigation has been extended to the electron microscopical level and the evaluation of the above changes is in progress.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02357-02 LNNS						
PERIOD COVERED October 1, 1970 to September 30, 1980								
TITLE OF PROJECT (80 characters or less) The therapeutic γ -hydroxybutyrate effect on experimental cerebral ischemia in Mongolian gerbils. Former title: The therapeutic chemical effect on experimental cerebral ischemia in Mongolian gerbils								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: W. D. Rausch</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: W. D. Rausch	Visiting Fellow	LNNS NINCDS
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COOPERATING UNITS (if any) None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: <div style="text-align: center;">0.4</div>	PROFESSIONAL: <div style="text-align: center;">0.2</div>	OTHER: <div style="text-align: center;">0.2</div>						
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
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SUMMARY OF WORK (200 words or less - underline keywords) The preliminary investigation of the effect of γ -hydroxybutyrate (GHB) on the neurotransmitters' synthesizing enzymes has shown that GHB stimulates the recovery of tyrosine and tryptophan hydroxylase and prevents the drop of dopa decarboxylase activities in the brains of ischemic gerbils.								

Project Description:

Objectives: Recently we have shown that the naturally occurring central nervous system (CNS) depressants, γ -hydroxybutyrate (GHB) and its lactone γ -butyrolactone (GBL), modified cerebral ischemia as was manifested by amelioration of cerebral metabolites and edema changes occurring in the ischemic gerbils (Smialek, Klatzo and Spatz, in: Cerebral Vascular Disease 2, Excerpta Medica, 1979; Abe, Abe, Klatzo and Spatz, in: Advances in Neurology, Vol. 28. Brain Edema, Raven Press, 1980). In order to elucidate the mechanism of GHB or GBL action in ischemic brain, we investigated the enzymes involved in the synthesis and metabolism of neurotransmitters which are affected by GHB or GBL under normal conditions (Walters and Roth, in: Neuroregulators and Psychiatric Disorders, Oxford University Press, 1977).

Methods Employed: Fifteen minutes of bilateral common carotid artery occlusion with and without release served as a model for the production of cerebral ischemia in gerbils. The treatment consisted of a single injection of GHB (500 mg/kg) 2 minutes prior to occlusion (preischemic group) and 3 hours after the release of occlusion (postischemic group). Sham-operated and GHB-injected as well as untreated gerbils served as controls. Tyrosine hydroxylase, tryptophan hydroxylase and dopa decarboxylase were assayed by either radiolabeled or spectrofluorescent techniques (Nagatsu, T., Biochemistry of Catecholamines, University of Tokyo Press, 1973).

Major Findings: These experiments are still in a preliminary stage. Nevertheless, the following trends of the GHB effects on the tested enzymes were observed in the ischemic brain: 1) stimulation of the recovery of tyrosine and tryptophan hydroxylase activities, and 2) prevention of dopa decarboxylase activity reduction occurring in the untreated ischemic animals. These results suggest that the beneficial action of the endogenous CNS depressants on cerebral ischemia might be the result of cerebral neurotransmitters' level stabilization.

Significance to Biomedical Research and the Program of the Institute: The beneficial therapeutic effect of the naturally occurring CNS depressants in the experimentally induced ischemia indicates that these substances might be useful clinically following the complete evaluation of these agents in various models of cerebral ischemia.

Proposed Course of the Project: The study of the effect of GBL and GHB on catecholamine and other metabolic pathways in the brain will be continued in cerebral ischemia in order to elucidate the pathophysiological mechanism of their beneficial action.

Publications: See Project No. Z01 NS 02360-02 LNNS

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02358-02 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) The postischemic effect on the uptake and metabolism of monoamines in isolated cerebral capillaries														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">T. Abe</td> <td style="width: 40%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	T. Abe	Visiting Fellow	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
PI:	T. Abe	Visiting Fellow	LNNS NINCDS											
Other:	K. Abe	Visiting Fellow	LNNS NINCDS											
	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS											
COOPERATING UNITS (if any) B. B. Mrsulja, Institute of Biochemistry, Faculty of Medicine, Belgrade, Yugoslavia														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.2</div>												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS							
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SUMMARY OF WORK (200 words or less - underline keywords) The <u>cerebral microvessels</u> isolated from brains of gerbils subjected to 15 minutes of <u>complete cerebral ischemia</u> and various periods of <u>recovery</u> had shown a selectively altered cerebral microvascular accumulation of monoamines in postischemia while the enzymes involved in the degradation of the amines were affected by ischemia and postischemia. The modified function of the isolated cerebral microvessels was closely related to the blood-brain barrier changes for monoamines observed in parallel studies.														

Project Description:

Objectives: The isolated cerebral microvessels have been useful in the investigation of transport processes occurring in the blood brain level (Spatz et al., in Pathophysiology of Cerebral Energy Metabolism, New York, Plenum Press 1979, pp. 143-153). Recently we have shown a postischemic selective increase in the brain uptake of monoamines which under normal conditions don't penetrate the blood-brain barrier (BBB). To elucidate the mechanism responsible for these observations, the changes in the uptake of the amines and the activity of the degrading enzymes were investigated in microvessels isolated from ischemic and postischemic brain.

Methods Employed: The experiments were designed to coincide with the observed presence and absence of increased BBB permeability to 5-HT and NE. The correlative studies comprised: a) the uptake of the radiolabeled metabolizable amines (5-HT and NE) and the labeled nonmetabolizable NE analogue, metaraminol (M), and b) the determination of catechol-O-methyl transferase (COMT) and monoamine oxidase [total MAO, (A) and (B) forms] levels in the cerebral microvessels.

Major Findings: The increase in the capillary uptake of 5-HT occurred prior to that of NE and M, in concurrence with the findings of selective postischemic BBB leakage. At the same time (24 hours recovery), the depression of MAO activity was maximal and the elevation of COMT activity was considerably smaller than that of the other experimental periods (15 minutes without release and with 72 hours recovery). Thus, the most marked reduction in the MAO activity coincided with the increased capillary uptake of 5-HT and increased passage of 5-HT from blood to brain. Subsequently, at 72 hours of postischemia when the BBB permeability was altered for 5-HT and NE, the uptake of each amine, the metabolizable (5-HT and NE) and the nonmetabolizable (M), in the microvessels was above normal levels. At this time, the activity of COMT was fourteenfold higher and that of MAO 30% lower in the experimental and control microvessels. A similarly altered activity of both enzymes but not of the amines' uptake was observed in cerebral microvessels after ischemia only. These findings suggest that at this particular time both the disturbed transport and metabolism might be responsible for the observed increased passage of the amines across the BBB.

Significance to Biomedical Research and the Program of the Institute: Based on our investigation, the cerebral capillaries are useful for the study of some parameters of brain transport phenomena occurring in both physiological and pathological conditions. The knowledge of the functional state of cerebral capillaries is extremely important, since it may either be responsible for many metabolic changes occurring in the brain and/or it may reflect the altered metabolic state of the brain in many disease processes.

Proposed Course of the Project: This project is incomplete as yet. A similar model will be used for evaluation of other catecholamines' capillary uptake as well as release studies in ischemic and postischemic gerbils. Moreover the capillary uptake of monoamines will be correlated with the activity of enzymes involved in the synthesis and metabolism of the respective substrate. This work was presented at the Second International Belgrade Symposium on Pathophysiology of Cerebral Metabolism, September 1979.

Publications:

Abe, T., Abe, K., Mičić, D., Djuričić, B. M., Mrsulja, B. B., and Spatz, M.: Studies on the blood-brain barrier (BBB) to monoamines. In Spatz, M., Mrsulja, B. B., Rakić, Lj. M., and Lust, W. D. (Eds.): Circulatory and Developmental Aspects of Brain Metabolism. New York, Plenum Press, pp. 215-223.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02359-02 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) ³ H Metaraminol uptake in isolated cerebral capillaries														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">T. Abe</td> <td style="width: 30%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	T. Abe	Visiting Fellow	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
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Other:	K. Abe	Visiting Fellow	LNNS NINCDS											
	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS											
COOPERATING UNITS (if any) None														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0.6</div>	PROFESSIONAL: <div style="text-align: center;">0.4</div>	OTHER: <div style="text-align: center;">0.2</div>												
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<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS													
SUMMARY OF WORK (200 words or less - underline keywords) The elucidation of the mechanism involved in the <u>mongamine uptake</u> in the <u>cerebral capillaries</u> has been investigated by using <u>³H metaraminol</u> , a nor-epinephrine analogue which is not metabolized by either COMT or MAO. These studies have shown that the ³ H metaraminol is taken up by K ⁺ - and Na ⁺ -dependent, carrier-mediated process in the isolated cerebral microvessels similar to the one reported in the neurons. This project is completed.														

Project Description:

Objectives: Norepinephrine, which doesn't cross the blood-brain barrier, can be taken up and metabolized in isolated cerebral microvessels showing features of both extraneuronal and neuronal uptake. In order to elucidate further the monoamine's uptake in cerebral microvessels, we investigated the uptake of ^3H metaraminol, a norepinephrine analogue which is not metabolized by either MAO or COMT.

Methods Employed: The isolated cerebral capillaries were incubated with ^3H metaraminol in Ringer's solution containing .1% albumin (pH 7.4) alone or with various concentrations of unlabeled (cold) metaraminol, L-dopa, dopamine, 5-hydroxydopamine, 6-hydroxydopamine, norepinephrine, 5-hydroxytryptamine, normetanephrine and metanephrine. The effect of metabolic inhibitors and adrenergic blocking agents on the uptake of ^3H metaraminol was also evaluated under physiological conditions.

Major Findings: The capillary uptake of ^3H metaraminol increased with the time of incubation (30 sec-15 min). The uptake was found to be saturable, because it could be inhibited by addition of unlabeled metarminol in increasing concentrations to the incubation media containing the labeled substance. The estimated k_m was $1.1 \mu\text{M}$. The accumulation of ^3H metaraminol in the capillaries was stimulated by K^+ and Na^+ and inhibited by hypothermia, ouabain, KCN, DPN, adrenergic blocking agents (imipramine, propanolol, dichloroisoproterenol and phentolamine). Moreover, the ^3H metaraminol capillary uptake was competitively inhibited by arterenol (k_i), 5-hydroxytryptamine (k_i) and cross-inhibited by dopamine, 6-hydroxydopamine, 5-hydroxydopamine, L-dopa but not by normetanephrine or metanephrine.

Significance to Biomedical Research and the Program of the Institute: These results indicate that ^3H metaraminol is taken up by K^+ - and Na^+ -dependent, carrier-mediated mechanism (which may be shared by other monoamines) in the cerebral microvessels. This process appears to be similar to the one described for neuronal monoamine uptake especially since extraneuronal uptake of amines was reported to be insensitive to metaraminol but sensitive to normetanephrine and metanephrine.

Proposed Course of the Project: These investigations are completed and this work was presented at the Neuroscience meeting in the fall of 1979 and at the Symposium on Cerebral Microvasculature: Investigation of the Blood Brain Barrier, in Galveston, July 1979.

Publications: See Project No. Z01 NS 02324-03 LNNS

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02360-02 LNNS																
PERIOD COVERED October 1, 1979 to September 30, 1980																		
TITLE OF PROJECT (80 characters or less) The effect of central nervous system depressants on ischemic cerebral edema of gerbils																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">K. Abe</td> <td style="width: 40%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>T. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	K. Abe	Visiting Fellow	LNNS NINCDS	Other:	T. Abe	Visiting Fellow	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
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COOPERATING UNITS (if any). None																		
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INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.2</div>																
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>																		
SUMMARY OF WORK (200 words or less - underline keywords) The correlation of either gamma-hydroxybutyrate (GHB) or gamma-butyrolactone (GBL) with pentobarbital (Pb) treatment (prior to or after the induction of ischemic brain edema) has shown that the endogenous substances were more effective than the exogenous central nervous system depressants in suppressing the brain edema.																		

Project Description:

Objectives: Recently, we had shown that the outcome of cerebral ischemia can be modified by treatment with the naturally occurring central nervous system depressants [γ -hydroxybutyrate (GHB) or γ -butyrolactone (GBL)]. Among the beneficial effects of pre- and postischemic GHB and GBL treatment was the reduction of cerebral edema observed in gerbils subjected to bilateral cerebral ischemia. Since the barbiturates have also been known to ameliorate various types of brain edema, we extended our studies to pentobarbital (Pb) and evaluated its effect on ischemic cerebral edema in the same model.

Methods Employed: Fifteen minutes of bilateral common carotid artery occlusion served as a model for the production of cerebral ischemic edema in gerbils. The treatment consisted of a single intravenous injection of either GHB (500 mg/kg) or GBL (300 mg/kg) or Pb (55 mg/kg) either 2 minutes prior to or 2 or 3 hours following the occlusion. The changes in the blood-brain barrier permeability to radiolabeled sucrose and 5-hydroxytryptamine (5-HT) were correlated with the changes in the specific gravity of cortex, hippocampus and basal ganglia in order to assess the extent of edema formation and its susceptibility to the action of CNS depressants.

Major Findings: GHB was the only substance which prevented the drop of specific gravity in the hippocampus. The progression of this drop in the hippocampus as well as in the basal ganglia and cortex was ameliorated to a greater degree by GHB and GBL than by Pb pretreatment. Similarly the abnormal passage of sucrose and 5-HT across the BBB seen in the untreated animals was more effectively reduced with GHB and GBL than with the Pb preischemic treatment. This difference in the GHB or GBL and the Pb attenuation of the cerebral specific gravity and BBB permeability was also seen when these substances were given 2-3 hours after the restoration of cerebral blood supply.

These findings are in agreement with the observed greater survival rate of gerbils subjected to preischemic and postischemic GHB and GBL than those of Pb treatment. Moreover, the different influence of these agents on brain edema is consistent with the better preservation and faster recovery of energy and carbohydrate metabolism seen in the brains of gerbils injected prior to the induction of ischemia, with either GHB or GBL than those with Pb.

Significance to Biomedical Research and the Program of the Institute: The evaluation of chemicals which may modify cerebral ischemic sequelae such as cerebral edema is of utmost importance clinically. Further elucidation of the therapeutic pathomechanism involved in the beneficial effect of central nervous system depressants in experimental cerebral ischemia and edema will be useful in considering the safety of such compounds for human treatment. A part of this project will be presented at the 10th International Symposium on Cerebral Vascular Disease in the fall of 1980.

Proposed Course of the Project: This investigation is still in progress. The beneficial therapeutic effect of GBL and GHB will be compared with the effect of exogenous central nervous system depressants such as pentobarbital on other models of edema for a better assessment of their usefulness as therapeutic agents.

Publications:

Smialek, M., Klatzo, I., and Spatz, M.: The therapeutic effect on experimental cerebral ischemia in Mongolian gerbils. In Meyer, J.S., Lechner, H., and Reivich, M. (Eds.): Cerebral Vascular Disease 2. Amsterdam-Oxford, Excerpta Medica, 1979, pp. 186-192.

Abe, K., Abe, T., Klatzo, I., and Spatz, M.: The effect of endogenous central nervous system depressants in ischemic cerebral edema of gerbils. In Cervós-Navarro, J., and Ferszt, R. (Eds.): Advances in Neurology, Vol. 28. Brain Edema. New York, Raven Press, 1980, pp. 429-442.

Klatzo, I., and Spatz, M.: Experimental cerebral ischemia. In Davison, A.N., and Thompson, R.H.S. (Eds.): The Molecular Basis of Neuropathology. London, Edward Arnold Publishers, 1980 (in press).

Project Description:

Objectives: The aim of this study has been to investigate the permeability of the blood-brain barrier (BBB) in bilateral cerebral ischemia, since unilateral ischemia produced selective and diverse effects of BBB functions in the affected cerebral hemisphere (Spatz, Fujimoto, Go, in Dynamics of Brain Edema, Berlin-Heidelberg, Springer Verlag 1976, pp. 181-186).

Methods Employed: Several groups of adult gerbils were subjected to bilateral common carotid artery clipping for 3, 6 and 15 minutes with and without clip release. The following tracers have been used so far for the evaluation of the BBB: NaFl, Evans blue, ^{14}C sucrose, ^3H norepinephrine (NE), ^3H 5-hydroxytryptamine (5-HT) and ^3H dextran (Mol. weight 60,000).

Major Findings: The BBB permeability was found to be intact to NaFl, sucrose and Evans blue during the 3, 6 and 15 minutes of bilateral common carotid artery occlusion. However, 30-50% of gerbils showed an increased BBB permeability to NaFl and sucrose after 30 minutes of reestablished cerebral circulation. The incidence of increased BBB permeability to NaFl and sucrose depended on the duration of ischemia and was not seen in animals with the clip released for 3 and 5 hours following occlusion for 3 and 6 minutes, respectively. In 15 minutes of bilateral occlusion, the greatest incidence of BBB sucrose leakage was seen after 3 and 7 days of recovery, while that of dextran was at 7 days of clip release. Moreover, selective BBB permeability changes to monoamines were observed in the recovery of 24 hours (5-HT) and 72 hours (NE).

Significance to Biomedical Research and the Program of the Institute: The basic comprehension of the blood-brain barrier behavior and function concerned with the passage of nutrient and non-nutrient substances from blood to brain following cerebral ischemia is of major importance (1) for the understanding of the mechanism responsible for the development of ischemic edema, as well as elucidating other pathophysiological processes in cerebrovascular disease and many other neurological disorders, and (2) for selecting the best therapeutic approach to a given disease.

Proposed Course of the Project: These investigations are still incomplete and require the evaluation of the BBB permeability changes and/or recovery at late periods of recirculation after 15 minutes of bilateral cerebral ischemia.

Publications:

See Project No. Z01 NS 02322-03 LNNS and Project No. Z01 NS 02356-02 LNNS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01995-08 LNNS																				
PERIOD COVERED October 1, 1979 to September 30, 1980																						
TITLE OF PROJECT (80 characters or less) Morphological studies of myelin formation, breakdown and regeneration																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">H. deF. Webster</td> <td style="width: 30%;">Associate Chief</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>N. Sternberger</td> <td>Expert</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>Y. Itoyama</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>B. D. Trapp</td> <td>Staff Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>R. H. Quarles</td> <td>Chief, Myelin and Brain Development Section</td> <td>DMNB NINCDS</td> </tr> </table>			PI:	H. deF. Webster	Associate Chief	LNNS NINCDS	Other:	N. Sternberger	Expert	LNNS NINCDS		Y. Itoyama	Visiting Fellow	LNNS NINCDS		B. D. Trapp	Staff Fellow	LNNS NINCDS		R. H. Quarles	Chief, Myelin and Brain Development Section	DMNB NINCDS
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	R. H. Quarles	Chief, Myelin and Brain Development Section	DMNB NINCDS																			
COOPERATING UNITS (if any) Developmental and Metabolic Neurology Branch, NINCDS; Department of Neurology, Johns Hopkins Medical School, Baltimore, Md., Department of Neuropathology and Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, Mass.																						
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																						
SECTION Section on Cellular Neuropathology																						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																						
TOTAL MANYEARS: <div style="text-align: center; font-size: 1.2em;">5.7</div>	PROFESSIONAL: <div style="text-align: center; font-size: 1.2em;">4.5</div>	OTHER: <div style="text-align: center; font-size: 1.2em;">1.2</div>																				
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SUMMARY OF WORK (200 words or less - underline keywords) <p>The long range goal of this project is to combine <u>immunocytochemical methods</u> with <u>light and electron microscopy</u> to study <u>cellular mechanisms of myelin formation, breakdown and regeneration</u>. Nervous tissues from experimental animals and patients with demyelinating disease have been studied in the following current projects: 1) Distribution of myelin-associated glycoprotein (MAG) and basic protein (BP) in hexachlorophene (HCP) intoxication, experimental allergic encephalomyelitis (EAE) and a genetic defect of myelin formation (jumpy mice). 2) Distribution of MAG, BP, and papova virus in progressive multifocal leukoencephalopathy and use of immunocytochemical methods to identify remyelination in multiple sclerosis lesions.</p>																						

Project Description:

Objectives: To study the distribution of MAG and BP in the central nervous system (CNS) of HCP intoxicated rat pups. 2) To compare the distribution of MAG and BP in jimpy mice and littermate controls during CNS myelin formation. 3) To induce EAE in Lewis rats and study the distribution of MAG and BP before and during CNS myelin breakdown. 4) To study the distribution of papova virus, oligodendroglial MAG, and myelin BP in progressive multifocal leukoencephalopathy (PML) lesions. 5) To study remyelination in multiple sclerosis (MS) lesions.

Methods Employed: 1) Eighteen-day-old rat pups were fed chow containing 500 PPM HCP and were perfused with aldehyde mixtures at ages 21-29 d. Blocks from some rats were sectioned on a vibratome and immunostained. Blocks from littermates were embedded in Epon, immunostained and adjacent sections were studied with the electron microscope. 2) Ten- to twenty-five-day-old jimpy mice and their littermate controls were fixed by perfusion before immunostaining vibratome or Epon embedded sections. Some Epon blocks were thin-sectioned also and studied with the electron microscope. 3) Spinal cord and complete Freund's adjuvant were used to induce EAE in Lewis rats and strain 13 guinea pigs. They were sacrificed by aldehyde perfusion before and during clinical illness; vibratome, paraffin and Epon sections were immunostained with MAG and BP antisera according to the peroxidase-antiperoxidase method. 4) Paraffin sections of CNS lesions from patients with PML were immunostained with antisera to P₀ protein (the major protein of peripheral nervous system myelin) and BP.

Major Findings: 1) During HCP intoxication, myelin sheaths become vacuolated but do not degenerate. In spite of severe vacuolation, BP distribution remained confined to myelin and the periaxonal distribution of MAG was unchanged also. When HCP was withdrawn, the myelin vacuoles became smaller and disappeared. 2) In jimpy mice, oligodendroglia are reduced in number and there is a severe deficit in CNS myelin formation. MAG antiserum only stained a few of the oligodendroglia that were present and the staining intensity was decreased. BP antiserum stained a higher proportion of oligodendroglial perikarya and ensheathing processes than MAG antiserum. Phase and electron microscopic study of these stained periaxonal regions showed that they were not compact myelin sheaths. 3) Early focal fragmentation of myelin sheaths was easier to identify with BP immunostaining than with Luxol Fast Blue or other histological methods. In more advanced lesions, fragmenting sheaths and demyelination were found beyond margins of perivascular infiltrates, especially in the gray matter. In zones of myelin fragmentation and breakdown, periaxonal staining by MAG antiserum was abnormal also. 4) In paraffin sections from a rapidly progressive case of PML, hyperimmune JC virus antiserum stained single oligodendroglia in white matter that appeared normal histologically and stained normally with BP and MAG antiserum. In zones surrounding areas of demyelination, virus-containing oligodendroglia were most numerous, MAG staining of periaxonal regions was decreased, but there was little change in myelin BP staining. There was much less JC virus staining in demyelinated regions where both MAG and BP staining were severely altered. 5) In paraffin sections of spinal cord blocks from control and multiple sclerosis patients, P₀ anti-

serum staining showed that in large MS plaques, there are peripheral myelin sheaths produced by Schwann cells. These peripheral sheaths do not contain P₂ (a protein that is restricted to large sheaths and is not found in regenerating sheaths). BP antiserum stained a few oligodendroglia at the margins of inactive plaques.

Significance to Biomedical Research and the Program of the Institute:

1) HCP intoxication, by splitting and vacuolating compact myelin reversibly, allows the persistence of MAG and its periaxonal localization to be convincingly demonstrated. Since MAG distribution is not changed and demyelination does not occur, perhaps MAG does have a role in oligodendroglial maintenance of MAG. 2) Our results in jimpys suggest that the oligodendroglia present can make BP and transport it to periaxonal locations for insertion into developing myelin sheaths. Oligodendroglial formation of MAG is much more impaired and may have a role in the failure of immature loose spirals to develop into compact sheaths that grow normally. 3) EAE is considered by many to be an experimental model for multiple sclerosis. However, in the demyelinating lesions that develop in Lewis rats sensitized with spinal cord, the distribution of MAG and BP changes is similar and unlike that found in multiple sclerosis. The differences suggest that in MS, the oligodendrocyte may be affected sooner or more severely than in this type of EAE. 4) Our observations are among the first to localize CNS viral antigens by immunostaining paraffin and epon sections. They also emphasize that abnormal MAG immunostaining may be an early sign of oligodendroglial disease that will lead to myelin breakdown. 5) Although remyelination of bare axons in MS plaques by Schwann cells and oligodendroglia probably is not extensive enough to contribute to functional recovery, its distribution can be studied by the techniques we have described.

Proposed Course of the Project: To be continued. The above findings were presented at annual meetings of the Society for Neuroscience, the American Society for Neurochemistry, and the American Association of Neuropathologists.

Publications:

Trapp, B. D., McIntyre, L. J., Quarles, R. H., Sternberger, N. H., and Webster, H. deF.: Immunocytochemical localization of rat PNS myelin proteins: P₂ protein is not a component of all PNS myelin sheaths. Proc. Natl. Acad. Sci. USA 76: 3552-3556, 1979.

Itoyama, Y., Sternberger, N. H., Kies, M. W., Cohen, S. R., Richardson, E. P., Jr., and Webster, H. deF.: Immunocytochemical method to identify myelin basic protein in oligodendroglia and myelin sheaths of the human nervous system. Ann. Neurol. 7: 157-166, 1980.

Itoyama, Y., Sternberger, N., Quarles, R., Webster, H. deF., Richardson, E. P., Jr., Cohen, S., and Moser, H. W.: Successful immunocytochemical localization of myelin components in paraffin sections of human nervous tissue with preliminary observations on multiple sclerosis and meta-chromatic leukodystrophy lesions. Trans. Amer. Neurol. Assoc. 103: 216-219, 1978.

Tabira, T., and Webster, H. deF.: E-PTA stains oligodendroglial surface membranes and microtubules in optic nerves during myelination. J. Neurol. Sci. 42: 215-227, 1979.

Bray, G. M., Cullen, M. J., Aguayo, A. J., and Rasminsky, M.: Node-like areas of intramembranous particles in the unensheathed axons of dystrophic mice. Neurosci. Letters 13: 203-208, 1979.

Itoyama, Y., Sternberger, N. H., Quarles, R. H., Cohen, S. R., Richardson, E. P., Jr., and Webster, H. deF.: Immunocytochemical observations on the distribution of myelin-associated glycoprotein and myelin basic protein in multiple sclerosis lesions. Ann. Neurol. 7: 167-177, 1980.

Trapp, B. D., and Richelson, E.: Usefulness of rotation-mediated aggregating cell cultures. In Spencer, P. S., and Schaumburg, H. (Eds.): Experimental and Clinical Neurotoxicology. Baltimore, Williams and Wilkins, 1980, pp. 803-819.

Webster, H. deF., Trapp, B. D., and Cullen, M. J.: Xenopus tadpoles: A useful model for studying cellular effects of neurotoxic compounds. In Spencer, P. S., and Schaumburg, H. (Eds.): Experimental and Clinical Neurotoxicology. Baltimore, Williams and Wilkins, 1980, pp. 775-787.

Webster, H. deF., and Sternberger, N. H.: Morphological features of myelin formation. In Baumann, N. (Ed.): Proceedings of Conference on Neurological Mutants Affecting Myelination, Research Tool in Neurobiology. Amsterdam, Elsevier, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01996-08 LNNS									
PERIOD COVERED October 1, 1979 to September 30, 1980											
TITLE OF PROJECT (80 characters or less) Membrane structure in CNS tissue and subcellular brain fractions											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 60%;">H. deF. Webster Associate Chief</td> <td style="width: 25%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>B. D. Trapp Staff Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>R. H. Quarles Chief, Myelin and Brain Development Section</td> <td>DMNB NINCDS</td> </tr> </table>			PI:	H. deF. Webster Associate Chief	LNNS NINCDS	Other:	B. D. Trapp Staff Fellow	LNNS NINCDS		R. H. Quarles Chief, Myelin and Brain Development Section	DMNB NINCDS
PI:	H. deF. Webster Associate Chief	LNNS NINCDS									
Other:	B. D. Trapp Staff Fellow	LNNS NINCDS									
	R. H. Quarles Chief, Myelin and Brain Development Section	DMNB NINCDS									
COOPERATING UNITS (if any) Developmental and Metabolic Neurology Branch, NINCDS; J. M. Matthieu, University of Lausanne School of Medicine, Lausanne, Switzerland; Department of Neurology, Johns Hopkins Medical School, Baltimore, Md.											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Cellular Neuropathology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>									
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>											
SUMMARY OF WORK (200 words or less - underline keywords) This project has been completed. Publication: Trapp, B. D., McIntyre, L. J., Quarles, R. H., Nonaka, G., Moser, A., Moser, H. W., and Webster, H. deF.: Biochemical characterization of myelin isolated from the central nervous system of <u>Xenopus</u> tadpoles. <u>J. Neurochem.</u> 34: 1241-1246, 1980.											

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01805-12 LNNS												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Membrane Structure and Cytosol Enzymes														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">J. J. Anders</td> <td style="width: 35%;">Guest Worker</td> <td style="width: 15%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>M. W. Brightman</td> <td>Head, Section on Neurocytology</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>D. Schmechel</td> <td>Research Associate</td> <td>LCS NIMH</td> </tr> </table>			PI:	J. J. Anders	Guest Worker	LNNS NINCDS	Other:	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS		D. Schmechel	Research Associate	LCS NIMH
PI:	J. J. Anders	Guest Worker	LNNS NINCDS											
Other:	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS											
	D. Schmechel	Research Associate	LCS NIMH											
COOPERATING UNITS (if any) Laboratory of Clinical Science, NIMH														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.5	OTHER: 0.1												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER												
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Manipulation of the number, size and distribution of particle assemblies</u> within the astrocytic cell membrane is best done <u>in vitro</u>. Primary cultures of astrocytes from 7-day-old rats are usually fed every 4 days, a schedule maintaining the fusiform shape of these cells and a paucity of assemblies. If the culture medium is "<u>conditioned</u>" by not feeding for 7 to 10 days, the cells become more normally <u>asteroid</u> in shape and the <u>number of assemblies increases</u>. The stimulus for these changes is not meningeal. Co-cultivation with cells from the pia-arachnoid results in cells no different from those derived from brains stripped of their leptomeninx. Small <u>neurons</u>, detected immunocytochemically, survive for at least 4 weeks and may be the source of the "<u>conditioning</u>" factor. In order to see whether the assemblies can be rearranged at all, the drastic treatment of denaturation with guanidine and urea was performed. Both <u>denaturants</u> caused <u>clumping</u> of assemblies into large aggregates to which background particles also adhered. Cytochalasin B did not rearrange the assemblies but <u>colchicine</u> caused an <u>augmentation</u> in their number and an <u>aggregation</u> comparable to denaturation, an effect suggesting that the assemblies may be <u>anchored</u> to the cytoplasmic matrix. </p>														

Project Description:

Objectives: To ascertain the function of assemblies by modifying their configuration, distribution and numbers in reactive astrocytes and in cultures. Project No. Z01 NS 02200-05 LNNS was incorporated herein.

Methods Employed: Primary cultures of astrocytes from dissociated brains of rats 7 days old are maintained for at least 3 weeks, with only intermittent feeding every 7 to 10 days. Denaturants, such as 2M guanidine and 1 to 8M urea are added to see whether changes in shape or distribution of the assembly particles can be detected. Redistribution might be restricted by anchoring microtubules in the cytoplasm. In order to test the possibility, 10 µg/ml of cytochalasin B or 10^{-4} M colchicine were added to the culture broth for 30 minutes.

Major Findings: When the cultures are not fed for 7 to 10 days, their cell shape changes from a fusiform to a more normal-appearing asteroid shape and the number of their assemblies is augmented. Some factor may be secreted into the culture fluid by small neurons that survive the explantation. Within denatured astrocytic membranes, background particles and assemblies become clumped. Although cytochalasin B had no effect, colchicine resulted in a remarkable crowding of assemblies, comparable to that in reactive astrocytes, and in clumping, comparable to denaturation.

Significance: The change in glial cell shape and assemblies may be influenced by neuronal secretion. The assemblies themselves may be anchored to cytoplasmic tubules. A mutual interaction between astrocyte and neuron is demonstrable from our work. A grant was awarded for this project by the Epilepsy Foundation of America for 1979 and 1980.

Proposed Course: To verify the neuronal-glial interaction and compare changes induced by other agents with those following denaturation and neuronal influences.

Publications: Anders, J. J., and Brightman, M.W.: Assemblies of particles in the cell membranes of developing, mature and reactive astrocytes. J. Neurocytol. 8: 777-795, 1979.

Schmechel, D. E., Brightman, M.W. and Barker, J. L.: Localization of neuron-specific enolase in mouse spinal neurons grown in tissue culture. Brain Res. 181: 391-400, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 02086-07 LNNS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regeneration in Peripheral and Central Nerves
Former title: Regeneration in Vertebrate Nerves

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Rosenstein	Guest Worker	LNNS NINCDS
Other:	D. Rubin	Guest Worker	LNNS NINCDS
	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Neuropathology and Neuroanatomical Sciences

SECTION

Section on Neurocytology

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.3

PROFESSIONAL:

1.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fragments of allografted superior cervical ganglion (SCG) have not been rejected from the rat's intact brain surfaces 2 years after transplantation. Within SCG grafts, norepinephrine (NE), detected by the chromaffin reaction, can be synthesized within ganglion cells. Numerous storage vesicles occupy axons and growing tips, indicating axoplasmic transport of NE. Schwann cells also contain vacuoles with reaction product, due to NE uptake by non-neuronal cells from the transplant's extracellular fluid. Iris, liver and pancreas from rats given 5-hydroxydopamine did not have reaction product in their lysosomes but the ganglion and Schwann cells did. Schwann cells from the graft invaded the cerebellum to act in concert with astroglia in enveloping neural elements - a "cooperation" hitherto unrecognized. SCG grafts over the area postrema evoked a predominantly glial rather than a neuronal migration. Sprouting of axon collaterals from central neurons of frog spinal cord is now being examined. Lumbar motoneurons receive afferents from (1) segmental dorsal roots and (2) lateral column fibers. Labeling the proximal stump of a cut thoracic nerve with peroxidase should distinguish the segmental supply from the unlabeled column sprouts.

Project Description:

Objectives: To establish the conditions for regeneration of both peripheral and central neurons and to characterize the stimulus from autonomic transplants that evoke anomalous migration of cerebellar tissue.

Methods: Rats with SCG allografts on the undamaged brain surface are fixed with chromate-aldehyde or prelabeled with the amine analogue, 5-hydroxy-dopamine for the detection of NE-containing structures at the fine structural level. In frogs, central axons from dorsal root ganglia are identified by soaking the proximal stump of cut thoracic nerves in horseradish peroxidase.

Significance: These results are the first to demonstrate that allografted autonomic ganglia can make, transport and store catecholamine. Schwann cells, by actively ensheathing CNS tissue, can acquire a functional role similar to that of astroglia.

Proposed Course: To further characterize the source of the factor attracting CNS neurons out of the brain and to ascertain the specificity of collateral sprouts ending on the motoneuron.

Publications: Rosenstein, J. M., and Brightman, M. W.: Regeneration and myelination in autonomic ganglia transplanted to intact brain surfaces. J. Neurocytol. 8: 359-379, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02144-06 LNNS									
PERIOD COVERED October 1, 1979 through September 30, 1980											
TITLE OF PROJECT (80 characters or less) Effects of Hypertension on the Permeability of Cerebral Endothelium to Proteins											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. W. Brightman</td> <td style="width: 33%;">Head, Section on Neurocytology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: K. Dorovini-Zis</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td> S. I. Rapoport</td> <td>Chief, Lab. of Neurosciences</td> <td>LNS NIA</td> </tr> </table>			PI: M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS	Other: K. Dorovini-Zis	Visiting Fellow	LNNS NINCDS	S. I. Rapoport	Chief, Lab. of Neurosciences	LNS NIA
PI: M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS									
Other: K. Dorovini-Zis	Visiting Fellow	LNNS NINCDS									
S. I. Rapoport	Chief, Lab. of Neurosciences	LNS NIA									
COOPERATING UNITS (if any) J. Robinson, Evanston Hospital, Evanston, Illinois Laboratory of Neurosciences, NIA											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Neurocytology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.5	OTHER: 0.2									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER									
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) <u>Serial plastic sections</u> were cut from brains of rats that had received horse-radish peroxidase (<u>HRP</u>) and lanthanum chloride (<u>La</u>) after infusion of <u>hyperosmotic arabinose</u> into the internal carotid artery. In a <u>few junctions</u> between <u>endothelial cells</u> of cerebral vessels, this smallest electron dense tracer, <u>ionic lanthanum</u> , could be followed <u>from the vessel lumen</u> to the perivascular <u>basal lamina</u> , a continuity indicating deformation of the junctions due to shrinkage of the endothelial cells. HRP did not appear to enter in this fashion. In order to see whether HRP passes across endothelium and choroid plexus epithelium actively, by vesicular transport, or passively, i.e., extracellularly via junctions, the <u>temperature of the brain</u> was reduced to 22-24°C by <u>hypothermia</u> . In these brains, the number of HRP-laden vesicles in the endothelium have yet to be evaluated, but the number in the <u>choroidal epithelium</u> was very low, a decrease indicating <u>in vivo</u> , that <u>protein uptake</u> is due to an <u>active</u> endocytosis and can be diminished or halted at low temperatures.											

Project Discription:

Objectives: To ascertain the mechanisms by which exogenous protein and salt cross the blood-brain barrier that has been lowered hyperosmotically.

Methods: 1.6M arabinose solution is infused into the internal carotid artery of rats, followed by HRP and by 5 mM lanthanum chloride (La). In about 9 rats, a small (~ 1mm thick) thermoprobe is inserted into one cerebral hemisphere for continuous recording of brain-core temperature and the above experiment is repeated.

Major Findings: In a few endothelial junctions, La can be traced from blood to abluminal side of vessels. Only a few endothelial vesicles were labeled. In choroid plexus epithelium, the vesicular uptake of peroxidase is markedly diminished during hypothermia.

Significance: It is important to establish the mechanisms whereby molecules and salts are transferred across once impermeable cerebral vessels, so that reversible, non-damaging methods of introducing such agents as anti-tumor drugs or enzymes across the barrier can be developed. So far, the hyperosmotic method appears to be the most useful.

Proposed Course: To evalute the cerebral endothelium from hypothermic rats to see whether endocytosis and thus protein exudation in these cells, as in the choroidal epithelium, is slowed or halted by hypothermia during hyperosmotic stimulus.

Publications: Anders, J. J., Dorovini-Zis, K., and Brightman M. W.: Endothelial and astrocytic cell membranes in relation to the composition of cerebral extracellular fluid. In Eisenberg, H. M. and Suddith, R. L. (Eds.): The Cerebral Microvasculature New York, Plenum Publishing Corp., 1980, pp. 193-211.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02145-06 LNNS								
PERIOD COVERED October 1, 1979 to September 30, 1980										
TITLE OF PROJECT (80 characters or less) Anterograde Movement of Exogenous Protein and Hydrolases within Neurosecretory Axons										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">R. D. Broadwell</td> <td style="width: 33%;">Staff Fellow</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>M. W. Brightman</td> <td>Head, Section on Neurocytology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	R. D. Broadwell	Staff Fellow	LNNS NINCDS	Other:	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS
PI:	R. D. Broadwell	Staff Fellow	LNNS NINCDS							
Other:	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS							
COOPERATING UNITS (if any) None										
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences										
SECTION Section on Neurocytology										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205										
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0								
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated. Results Have Been Published: Broadwell, R. D., Oliver, C. and Brightman, M.W.: Localization of neurophysin within organelles associated with protein synthesis and packaging in the hypothalamo- neurohypophysial system: An immunocytochemical study. <u>Proc. Natl. Acad. Sci. USA</u> 76: 5999-6003, 1979.										

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02200- 05 LNNS
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Freeze-Fracture of Cell Membranes Intercalated with Lipids		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: M. W. Brightman Head, Section on Neurocytology LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been incorporated with Project No. Z01 NS 01805-12 LNNS.		

Project Description:

Objectives: Synapses are sites where electrical signals pass between neurons or between neurons and muscle cells. This project seeks to establish the structural basis and mechanism of synaptic transmission in the central and peripheral nervous systems in both adult and immature animals, and in tissue cultured neurons and muscles.

Methods Employed: Tissues are prepared for freeze-fracturing or for freeze-substitution by a new technique which rapidly freezes tissue surfaces in one msec. Thus, tissue prepared for freeze-fracturing experiences no chemical treatment, while tissue prepared for sectioning is fixed at low temperatures in non-aqueous solvents. Our main purpose is to visualize the events which accompany and immediately follow transmitter secretion. A typical experiment consists of giving a nerve-muscle preparation a single shock and freezing it from 3 to 1000 msec later in preparation for either freeze-fracturing or freeze-substitution. The initial stages in secretion have also been studied in a similar manner in Limulus amebocytes (blood cells), a preparation chosen because the secretory granules are very large and the secretion proceeds precipitously after these cells contact endotoxin. Growth cones from cultured synpathetic ganglion cells (rat) are also being studied by freeze-substitution.

In order to localize calcium in muscle and neural tissues, they are rapid-frozen and then cryofixed in acetone containing oxalic acid. This method was developed by measuring the loss of $^{45}\text{Ca}^{++}$ from tissues during preparation to minimize loss, and by localizing calcium in frog muscles where its natural distribution is already known.

Other studies of synapses still depend on conventional freeze-fracture techniques using chemical fixatives. The giant synapse from the squid has been examined because its physiological condition can be defined so precisely. Squid axons are then put in an aldehyde fixative, frozen, freeze-fractured, and the resulting replicas of split membranes examined at high resolution in an electron microscope. A similar approach is also being used to study differences between synapses at fast and slow muscles in the frog, and the responses of these nerves and muscles to nerve resection. Another project has been initiated to examine the membrane ultrastructure of temperature sensitive neurological mutants affecting neuromuscular junctions in Drosophila.

Major Findings: By freeze-fracturing rapid frozen neuromuscular synapses, it has been possible to determine the fate of synaptic vesicle membrane after synaptic vesicles fuse with the presynaptic plasmalemma. In less than 0.1 sec, the vesicle membrane is completely flattened out into the plasmalemma. Components of the vesicle membrane, appearing as particles after freeze-fracturing, then spread out to be collected a second later into particle islands which are parts of the coated vesicle system. The final fate of these components of the vesicle membrane is to be reincorporated into synaptic

vesicles. This finding of particle recycling extends earlier work of the section showing that local recycling of synaptic vesicles replaces those lost during synaptic activity.

The initial stages in membrane interaction which lead to membrane fusion and exocytosis are so rapid at the frog neuromuscular junction that we turned to a preparation in which we could examine much larger secretory granules, and where we expected the initial stages of secretion to be more long-lived. Limulus amebocytes secrete precipitously within seconds after exposure to endotoxin, so the initiation of this process can be studied by freezing at different short intervals after application of endotoxin. The first change is a small perforation in the plasmalemma which rapidly widens, suggesting that exocytosis begins at a point rather than along a wide front of intermembrane contact.

The rapid freezing technique is also applicable to localizing calcium in tissues, provided the frozen tissue is subsequently cryofixed in the presence of oxalic acid. In muscle treated in this manner, we could detect no washout of calcium, and the calcium was localized with an electron probe at its expected positions in terminal cisterns of sarcoplasmic reticulum. We have applied this approach to stimulated synapses, where we found that the calcium which enters from the outside is sequestered in endoplasmic reticulum. We have also identified a similar system in amebocytes which sequesters the intracellular calcium after it has initiated the secretory events.

Comparative studies of other types of synapses were made using either rapid-freezing or conventional fixation to prepare them for freeze-fracturing. The giant synapse in the squid was shown to have well-defined synaptic vesicle release areas. We have serial-sectioned whole squid synapses to determine the numbers of membrane particles associated with release areas (1.1×10^7) and shown that this number yields a reasonable single particle conductance when each particle is assumed to be a single calcium channel, which substantiates our earlier suggestions that these particles are, in fact, calcium channels.

The extent of local recycling at the frog neuromuscular junction was measured by stimulating isolated synapses for up to 48 hours and then evaluating depletion of synaptic vesicles and other membranes. No depletion of synaptic vesicles has been found, even in preparations where axoplasmic supplies of membrane were blocked, either by initiating axoplasmic transport with colchicine or by ligating the nerve near the muscle. This preparation is now being studied with rapid-freezing and freeze substitution after stimulation in ferritin in order to see how new synaptic vesicles are formed once synaptic membranes are recovered.

The responses of nerve terminals to nerve resection are also being studied with conventional freeze-fracture techniques. The results suggest that at the moment when evoked transmitter release fails, the synaptic active zone particles disperse, and that spontaneous release is blocked only when the nerve is engulfed by Schwann cells, several hours later.

A scanning electron microscope was used to reveal the structural organization of the true outer surfaces of cells in nerve muscle preparations. We have developed a technique for chemically separating solid tissues in order to make them amenable to this form of examination. This technique is now being applied to examine the early responses of satellite cells in frog muscle to muscle injury. We are also beginning to apply it to study the Schwann cell response to nerve injury, and to determine the usefulness of this method as a tool for examining diseases affecting human muscle.

Finally, freeze-fracture at ultra low temperatures is being performed on squid axons to look for rapid changes in membrane structure subsequent to nerve impulses.

Significance to Biomedical Research and the Program of the Institute:

One of the most immediately practical aspects of these studies on synapses is that they define the normal structure of various types of synapses in a variety of functional states. This knowledge will permit distinction between normal and pathological, as well as between resting and active synapses, with the electron microscope. In diseases involving peripheral nerve-muscle synapses, it becomes possible to distinguish pathological states from changes resulting from increased or decreased activity. Our new studies on the development and degeneration at synapses may reveal, on a cellular level, why development or repair of synaptic systems is sometimes unsuccessful. Finally, our new directions in understanding how cells handle calcium will make it possible to study how these systems interact with the wide variety of drugs and diseases which affect our nervous system.

Our program of developing and adapting the rapid freezing, scanning, and freeze-fracture technique to study neural structure has been helpful to other program areas of NINCDS, as evidenced by the fact that major programs in neuroviruses, otolaryngology, and multiple sclerosis have found it important to make, with our assistance, major commitments to setting up facilities to perform research with this technique. In every instance, their primary investigators were trained in this technique in the Section on Functional Neuroanatomy.

Proposed Course of the Project: Much of the work outlined above is currently being prepared for publication, or has been submitted. The final work on rapid changes in frog neuromuscular synapses which accompany transmitter release is finished and the manuscript submitted. The work on the initial step in secretion in amoebocytes is being readied for publication.

A major new direction is to extend the rapid freezing and freeze-substitution techniques to new areas of synaptic and membrane physiology. In particular, we will take advantage of a new method we have developed to localize calcium to see how it is stored and released in a variety of neural tissues. We expect that the analytical work on calcium distribution will be greatly aided by our new high resolution analytical scanning transmission electron microscope which is almost functional. A second direction is to use

the scanning electron microscope and the freeze-fracture technique to study developing and degenerating synapses. A third direction is to use the rapid freezing in conjunction with high resolution freeze-fracture technique to look for configurational changes in intramembrane proteins during neural activity.

Publications:

Reese, T.S., and Heuser, J.E.: Changes in the structure of presynaptic membranes during transmitter secretion. In Hall, Z.W., and Otsuka, M. (Eds.): Neurobiology of Chemical Transmission. New York, John Wiley, 1979, pp. 3-11.

Cohen, S.A., and Pumplin, D.W.: Clusters of intramembrane particles associated with binding sites for α -bungarotoxin in cultured chick myotubes. J. Cell Biol. 82: 494-516, 1979.

Rees, R., and Reese, T.: New structural features of freeze-substituted neuritic growth cones. Neuroscience, (in press).

Smith, J.E., and Reese, T.: Use of aldehyde fixative to determine the rate of synaptic transmitter release. J. Exp. Biol. (in press).

Ornberg, R., and Reese, T.: A freeze-substitution method for localizing divalent cations: examples from secretory systems. Fed. Proc. (in press).

Carpenter, D., and Reese, T.: An overview of synaptic transmission. In: Siegel, G., Albers, R.W., Katzman, R., and Agranoff, B. (Eds.): Basic Neurochemistry, 3rd Edition. Boston, Little Brown and Company, Chapter 8, (in press).

Lynch, K.: Stimulation-induced reduction of large dense core vesicle numbers in cholinergic motor nerve endings. Brain Res. 194: 249-254, 1980.

Heuser, J.E., and Reese, T.S.: Structural changes following transmitter release at the frog neuromuscular junction. J. Cell Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 02284-04 LNNS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (60 characters or less)

Improvement of current methods of fixation by perfusion for preservation of glycogen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Cammermeyer Head, Section on Exp. Neuropath. LNNS NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Neuropathology and Neuroanatomical Sciences

SECTION

Section on Experimental Neuropathology

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The histochemical demonstration of glycogen was intensified after utilization of a perfusion procedure in which various recommendations were incorporated.

Project Description:

Objectives: To obtain consistent glycogen reaction in neurons and astrocytes for electron microscopic studies.

Methods Employed: The neuronal glycogen reaction is tested in animals which in narcosis were subjected to artificial respiration and fixation by perfusion with solutions containing inhibitors of glycolysis. Paraffin sections treated with dimedone are stained with periodic acid Schiff. Plastic embedded material is prepared for electron microscopic studies.

Major Findings: (1) Glycogen is demonstrable in perikarya of cortical neurons in which it has not previously been recognized .

(2) Glycogen is discernible in perikarya of Purkinje cells in which this has not been feasible except under unusual circumstances.

(3) Glycogen is distributed in a normal manner diffusely through perikarya of Purkinje cells situated in deep parts of the cerebellum.

(4) The glycogen staining is intensified as an expression of an improved preservation of this material by this new procedure of fixation.

(5) Inadequate fixation affects glycogen in neurons more than that in astrocytes.

(6) Although epinephrine, injected intracardially before perfusion, is essential for preservation of glycogen in neurons by directing flow of perfusates to the brain exclusively, it reduces content of glycogen in astrocytes.

Significance to Biomedical Research and the Program of the Institute: A method to improve preservation of glycogen is needed in order to estimate correctly the content of this substance in neurons under different experimental conditions. Also it may provide a basis to determine whether by changing the content of glycogen the vulnerability of neurons to experimental insults can be modified.

Proposed Course of the Project: To analyze the material for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02285-04 LNNS									
PERIOD COVERED October 1, 1979 to September 30, 1980											
TITLE OF PROJECT (80 characters or less) Cliniconeuropathologic study of brains from Guam											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 35%;">PI: J. Cammermeyer</td> <td style="width: 35%;">Head, Section on Exp. Neuropath.</td> <td style="width: 30%;">LNNS NINCDS</td> </tr> <tr> <td>Other: D. C. Gajdusek</td> <td>Chief, Lab. CNS Studies</td> <td>CNSS NINCDS</td> </tr> <tr> <td>C. J. Gibbs, Jr.</td> <td>Assoc. Chief, Lab. CNS Studies</td> <td>CNSS NINCDS</td> </tr> </table>			PI: J. Cammermeyer	Head, Section on Exp. Neuropath.	LNNS NINCDS	Other: D. C. Gajdusek	Chief, Lab. CNS Studies	CNSS NINCDS	C. J. Gibbs, Jr.	Assoc. Chief, Lab. CNS Studies	CNSS NINCDS
PI: J. Cammermeyer	Head, Section on Exp. Neuropath.	LNNS NINCDS									
Other: D. C. Gajdusek	Chief, Lab. CNS Studies	CNSS NINCDS									
C. J. Gibbs, Jr.	Assoc. Chief, Lab. CNS Studies	CNSS NINCDS									
COOPERATING UNITS (if any) Laboratory of Central Nervous System Studies, NINCDS											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Experimental Neuropathology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input checked="" type="checkbox"/> (b) HUMAN TISSUES</td> <td><input type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input checked="" type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input checked="" type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER									
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) This project is terminated.											

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02286-04 LNNS
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Mechanism of cerebral hemorrhages		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J. Cammermeyer Head, Section on Exp. Neuropath. LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Experimental Neuropathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Petechial cerebral hemorrhages induced by oil embolism in material fixed by perfusion are compared with those in material fixed by immersion.		

Project Description:

Objectives: To assess morphologic distinctions between petechial hemorrhages in material fixed by immersion and those fixed by perfusion.

Methods Employed: Injection of fat in systemic circulation of cats. Fixation by perfusion or by immersion after varying postinjection intervals. Intracardial injection of India ink during the perfusion. Embedding in paraffin or plastic. Histologic techniques for staining of erythrocytes and vascular walls.

Major Findings: Petechial hemorrhages and larger hemorrhagic infarctions composed of fresh erythrocytes aggregated near sites of vascular ruptures. They are of different appearance after use of the two types of fixation.

Significance to Biomedical Research and the Program of the Institute: An assessment of the factors contributing to hemorrhages may help to determine whether these hemorrhages occur during life or whether they can be the cause of death. Formulation of therapeutic measures as well as interpretation of hemorrhages as the cause of death will be dictated by the results of morphologic studies. The question is whether interpretation based on experimental material fixed by perfusion can be applied to material fixed by immersion, as is the case for human material.

Proposed Course of the Project: To supplement the immersion fixed material with longer post-operative intervals.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 02362-02 LNNS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of dimethyl sulfoxide on the histochemical demonstration of glycogen in the perfusion fixed brain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Cammermeyer

Head, Section on Exp. Neuropath.

LNNS NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Neuropathology and Neuroanatomical Sciences

SECTION

Section on Experimental Neuropathology

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When normal Netherlands dwarf rabbits were perfused with dimethyl sulfoxide (DMSO)-containing solutions, the brains exhibited pericapillary foci with acute tissue destruction and perivenous areas in which neurons were filled with glycogen. Glycogen was also discernible in microglial cells and oligodendrocytes. Because of the irregular distribution of glycogen-filled cells this method of fixation is not recommended for systematic studies on the distribution of glycogen in normal and experimental animals.

Project Description:

Objectives: To prevent polarization of glycogen in Purkinje cells by adding a drug to the fixative which will enhance infiltration of tissues.

Methods Employed: Glycogen was stained by the dimedone periodic acid Schiff technique in paraffin sections from brain fixed by perfusion with a modified Bouin's solution mixed with dimethyl sulfoxide in varying concentrations.

Major Findings: Irregular perivenous areas contain neurons filled with glycogen.

Purkinje cells do not display polarization of glycogen.

Glycogen is demonstrated in cerebellar granule cells, oligodendrocytes and microglial cells as well as astrocytes.

Significance to Biomedical Research and the Program of the Institute: The adoption of a special fixative in which DMSO is added made it possible to demonstrate glycogen to a degree not previously seen except in extraordinary conditions of hibernation, recovery from narcosis, X-irradiation and seasonal variations. These observations were typical of the Netherlands dwarf rabbit but they were not reproducible in the conventional rabbit or other animals. The method is instructive for investigations on qualitative characteristics of glycogen in animals but not for systematic studies in normal or experimental animals. The mode of action of DMSO remains enigmatic.

Proposed Course of the Project: To document the effect of DMSO on the speed of fixation in brains from animals of different ages.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 02363-02 LNNS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Segmental dendritic shrinkage and argentophilia in pericapillary lesions induced by dimethyl sulfoxide (DMSO) included in perfusates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Cammermeyer

Head, Section on Exp. Neuropath.

LNNS NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Neuropathology and Neuroanatomical Sciences

SECTION

Section on Experimental Neuropathology

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is terminated and the results have been published.

Cammermeyer, J.: Segmental shrinkage and argentophilia of dendrons after perfusion with dimethyl sulfoxide (DMSO)-containing solutions. Exp. Neurol. 67: 621-632, 1980.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neural Control, Intramural Research Program
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980
Laboratory of Neural Control, Intramural Research Program
National Institute of Neurological and Communicative Disorders and Stroke

Robert E. Burke, M.D., Chief

Introduction

Research work in the Laboratory of Neural Control (LNLC) primarily includes studies of the central and peripheral nervous system mechanisms involved in the control of movement in mammals. Emphasis is on those central nervous system structures that contain the neural organizations that produce the final stage of motor output - i.e., the spinal cord and those regions of the brain stem and cerebral cortex which project directly to the spinal cord. Several projects deal with the neurobiology of motoneurons and motor units, which are the functional quantum elements of motor output, and with afferent neurons that deliver sensory input and proprioceptive feedback information into the spinal cord to control motor unit activity.

A wide variety of technical approaches have been used in LNLC projects, including conventional electrophysiological, neuroanatomical and histochemical methods, as well as quite novel techniques, many developed within LNLC, for recording neural and mechanical data from awake, intact animals that are comfortable and either free to move or moving with minimal restraint. Both cats and monkeys are used in this research. Many of the newer techniques for recording neural activity in intact animals have derived from the long-standing interest of LNLC staff members in the problem of developing workable neural prostheses to aid the neurologically handicapped patient. A brisk interchange of ideas and information in this area exists between LNLC staff and staff members of the Fundamental Neurosciences Program of NINCDS, as well as with other groups in this country and abroad.

Present Organization

During FY 1980, the staff of the Laboratory of Neural Control (LNLC) has consisted of up to 14 professional scientists, including four permanent senior scientists (two M.D. and two Ph.D.), one Visiting Associate (D.D.S.), and seven post-doctoral fellows, including one Staff Fellow (Ph.D.), two Visiting Fellows (Ph.D.) and four (one M.D.-Ph.D and three Ph.D.) with other outside support. One scientist (Ph.D.) from the Department of Neurology, University of Maryland, has been worked in LNLC full time on an Intergovernment Personnel Act Assignment and another (Ph.D.) worked part time during a sabbatical leave from the University of Washington, Seattle. The permanent staff also includes three senior support personnel (two engineers and one physiologist), and the laboratory secretary; the non-permanent, part time staff includes a Junior Fellow (in Electronic Engineering) and a Laboratory Aide. One scientist from the Department of Neurology at the University of Maryland has collaborated with LNLC members as a Guest Worker on a part-time basis.

The staff members of LNLC have, in various combinations, backgrounds in neurophysiology, clinical medicine and neurology, and in biomedical engineering and computer sciences. Several staff members also have considerable expertise with biomaterials and techniques for fabrication of devices designed for chronic implantation. There is a great deal of interaction and interchange of ideas within

the LNLC group and staff members frequently collaborate with one another across formal "project" lines. In order to facilitate this interchange, LNLC is not divided into formal Sections but the research effort can be described under four general headings, with divisions based on methodological approach:

1. Research involving more or less conventional electrophysiological techniques and directed toward clarifying aspects of the cellular physiology and neuronal circuitry operating in the control of movement at the spinal cord level. This work is done largely using acute reduced preparations (both cats and monkeys), usually anesthetized or unanesthetized after decerebration under Halothane anesthesia. Some phases of this work also involve neuroanatomical techniques of cell labeling and pathway tracing with the exogenous protein tracer horseradish peroxidase (HRP), while other aspects involve study of muscles with conventional methods for muscle fiber histochemistry.

2. Research projects that utilize novel methods for recording the activity of individual neural elements in the central or peripheral nervous system of awake, intact animals (both cat and monkey) that are either free to move or able to perform motor tasks with minimal restraint. Some phases of this work also use techniques for recording kinesiological data, including limb position and joint angles from videotape records of movements of the whole animal or of individual limbs, and continuous recordings of muscle lengths and the forces produced by individual muscles obtained from transducers that are chronically implanted. Many of the methods and techniques involved in this work have been developed within LNLC and the necessary devices are designed and constructed by LNLC staff.

3. Theoretical and computer modeling studies of information processing in neural networks and ways in which data recorded from ensembles of neural elements can be analyzed and interpreted. This work is closely related to aspects of recording data from moving animals, since this involves analysis of multiple channels of neural records during non-stereotyped (i.e., imperfectly repeatable) movements.

4. Activities concerned directly with the development of new instruments and techniques, and the further refinement of existing methods, for recording and analyzing neurally-relevant data from intact, freely moving animals.

Project Summaries:

The functional output elements of the motor system are the motor units (a spinal motoneuron plus the set of muscle fibers, or "muscle unit", innervated by it). The central nervous system controls all motor acts by grading the numbers and identities of the several kinds of motor units ("recruitment") and by regulating the frequency of their firing ("rate coding" nor "rate modulation"). The project entitled "Intrinsic Properties of Motor Units" is designed to produce comprehensive descriptions of the electrophysiological, mechanical, morphological and histochemical characteristics of motor unit populations in particular muscles of the cat hindlimb. Cats are used because of the extensive background information already available about this animal and because they are suitable for a variety of experimental approaches, including investigations of motor behaviors such as locomotion and postural maintenance. Heterogeneous muscles in the cat hindlimb contain three major types of motor units (two varieties of fast twitch and one variety of slow twitch units), each with a distinctive set of mechanical and histochemical properties. The mechanisms underlying the differentiation and subsequent maintenance of the different motor unit types remain unknown. Previous work in LNLC has suggested that the unit types present in the normal, fully mature MG motor pool are resistant to alteration with respect to the characteristics that

serve to distinguish unit "types" when challenged by conditions such as compensatory hypertrophy, immobilization atrophy, and the atrophy that accompanies spastic paraplegia. A different picture emerges from when motor axons are cut and allowed to reinnervate their normal target muscle (self-reinnervation) or a foreign muscle (cross-reinnervation). During FY 1980, we completed a study of self- and cross-reinnervation using the flexor digitorum longus (FDL) and soleus (SOL) muscles of adult cats. The FDL muscle of the cat contains a heterogeneous mixture of all of the major motor unit types, of which about 90 percent are of the fast twitch types. In contrast, the SOL muscle is composed almost exclusively of slow twitch motor units. The motor units found in self-reinnervated muscles, both FDL and SOL, closely resemble those found in the normal muscles in all respects except for a wider than normal range in maximum force output. In particular, the usual motor unit types can be easily recognized and new types are not found. The histochemistry of individual self-reinnervated units (using glycogen-depletion to identify the muscle fibers of individual units) is exactly as expected from previous work on normal units, suggesting that the physiological-histochemical correlations found in normal muscles are completely re-established after successful self-reinnervation. However, the histochemical mosaic of the whole muscle is very much altered from normal in that fibers of individual units are located in abnormal positions. This strongly indicates that a large proportion of muscle fibers must be altered in their histochemistry, fiber size, etc., to conform to their new motoneuron. The self-reinnervation model provides one of the strongest pieces of evidence we have that the alpha motoneuron can specify in all details the characteristics of the muscle fibers innervated by it.

In the model of cross-reinnervation, the situation is much more complex. Both cross-reinnervation models tested (FDL \rightarrow SOL and SOL \rightarrow FDL) result in slow twitch, type S motor unit by physiological criteria (absence of "sag" property and fatigue index < 0.75 ; see previous Annual Reports). The type S motoneurons of the SOL nucleus produce virtually homogeneous populations of type S muscle units in cross-reinnervated FDL muscles, which are histochemically type I. Innervation of the homogeneous SOL muscle by the heterogeneous FDL motoneuron pool regularly produces type S muscle units that contract more rapidly than normal SOL muscle, although they are similar to normal SOL fibers histochemically. In the case of FDL \rightarrow SOL cross-reinnervation, there is an apparent failure of the expected conversion of slow twitch muscle fibers into fast twitch types. The results of the two crosses are thus asymmetrical. The major extension of this work in FY 1980 involved study of the dynamic activity of the FDL muscle in normal cats and in animals with self-reinnervation or cross-innervation by SOL motoneurons. Cross-reinnervated SOL muscles (with FDL motoneurons) were also studied using chronically-implanted transducers. The main findings are that normal FDL is used by the cat to a very variable extent in locomotion, with an activity pattern very different from the normal SOL. After self-reinnervation, the normal patterns continue without change. In cross-reinnervated muscles, the activity patterns are exactly those expected for the innervating motoneurons. Thus, there is no evidence for a change in functional usage to conform to that of the innervated muscle ("myotypic specification"). Further, the data indicate that the cross-innervated FDL is driven to extremes of activity never encountered normally, and this may well account for the completeness of "conversion" of its muscle units to type S. Conversely, the cross-innervated SOL has a much lower duty cycle than normal and this relative underuse seems correlated with the speedup of twitch contraction in these muscle units. However, it is absolutely clear that this extreme change in usage does not result in significant "conversion" of SOL muscle fibers to any variety of true fast twitch muscle.

Work done under the project entitled "Motor Control Systems in the Spinal Cord" is closely allied to the above, in that spinal segmental systems that project to alpha motoneurons are studied in relation to postsynaptic motor unit type. Previously, we have shown that a particular polysynaptic pathway conveys excitatory synaptic information from low threshold cutaneous afferents from distal skin regions (mainly in the sural nerve) primarily to ankle extensor motoneurons of the fast twitch type. This input system can alter the balance of functional thresholds to favor recruitment of the fast twitch units that are normally relatively high threshold. During FY 1980, we have been able to demonstrate that this excitatory cutaneous pathways receives convergence of excitatory control input from the rubrospinal and corticospinal tracts, suggesting that higher centers can adjust the relative balance of functional thresholds between slow and fast twitch units. A second aspect of this project concerns a study of the detailed anatomy of muscle stretch receptor (group Ia) afferents and of the contacts they establish on defined types of α motoneurons, using intracellular iontophoretic injection of horseradish peroxidase (HRP) into functionally identified group Ia afferents and subsequently into type-identified motoneurons. The results amply confirm the conclusion reached in this Laboratory a decade ago that Ia afferent contacts are established mainly on motoneuron dendrites, including their most distal regions. We are now building a catalog of afferent - motoneuron combinations to answer outstanding questions about the average number of terminations on individual motoneurons and whether the numbers and densities of Ia contacts differ in relation to motor unit type.

The work described above largely involves studies of motor mechanisms in anesthetized, immobile animals. The information and conceptual models obtained from such neurologically reduced preparations must be tested and supplemented by examination of neural activity in the intact, freely moving animal that can exhibit purposive behaviors. One approach to this now rapidly-developing area is represented by the project entitled "Neuron Activity in Locomotion". Much of this effort necessarily continues to be devoted to the design and application of methods that permit recording of activity in individual neural elements in freely moving cats, using chronically implanted electrodes in conjunction with other devices (length and force transducers, and videotape equipment) that permit monitoring the details of the studied movements. The available techniques now permit relatively reliable recording from functionally-identified sensory afferent neurons (muscle stretch receptors, Golgi tendon organ afferents, joint afferents, and cutaneous afferents of many modalities), as well as from identified alpha motoneuron axons in ventral roots, using chronically-implanted semi-microelectrodes. For technical reasons, this work has shifted to study of the muscles that extend the knee. Because of the wealth of background information about the peripheral characteristics and central connections of muscle receptor afferents, these have been of primary interest in the work to date. The observed patterns of afferent discharge are important in any attempt to understand the control of limb movement but they can in addition be used to infer activity in the fusimotor system that modulates spindle sensitivity. Major results to date indicate that the γ motor system, which controls the sensitivity of muscle stretch receptor afferents, operates in much more complex ways during normal movement than had been envisioned from conventional reflex physiology. There is evidence that some γ motoneurons are co-activated with the α motoneurons, particularly in some flexor muscles which shorten rapidly in stepping, while other spindle afferents, particularly in extensor muscles that undergo lengthening activation in stepping, exhibit less evidence of fusimotor bias. The ability to record from individual motor axons provides for the first time the possibility to follow the activity patterns of individual alpha motoneurons during normal movements. Microstimulation techniques are being developed

that appear promising to permit identification of active motor axons in terms of their muscle unit characteristics - i.e., identification by motor unit type. This will represent a major advance in motor system research if the methods can be made reliable and reproducible, since the approach is the only feasible method for direct study of recruitment patterns in relation to motor unit type in otherwise fully-characterized animal muscles. There is preliminary evidence that motor units within the same anatomical muscle (specifically to sartorius, a combined hip flexor and knee extensor) are specialized for either flexor or extensor function even though the muscle units can be recorded at a single location within the muscle. In addition, we have observed that the FDL and FHL muscles, which are strict anatomical synergists, can exhibit very different and even dissociated activity patterns during locomotion. These findings indicate that the classification of muscles and their motoneurons as "flexors" or "extensors" or even as "synergists" may be misleading unless based on direct evidence from moving animals.

The project entitled "Cortical Mechanisms of Voluntary Motor Control" is concerned mainly with studies of neural activity in particular regions of the cerebral cortex with proximate output to the spinal cord (the sensorimotor cortex and supplementary motor area) during voluntary motor behavior in awake intact animals. Both cats and monkeys are being studied. During FY 1980, the forearm and hand region of the monkey motor cortex (previously studied extensively using chronically-implanted electrodes) was explored with movable electrodes, using intracortical microstimulation (ICMS) and signal averaging methods to map the responsiveness of particular forearm muscles to highly localized cortical regions. Short trains of pulses at $< 20 \mu A$ intensity produce very variable patterns of excitation and/or inhibition of synergist and antagonist muscles. There is evidence that regions that produce inhibition of a given muscle tend to surround areas that produce its excitation. However, the cortical "colonies" that produce synaptic effects in a given muscle can be very extensive and often are discontinuous. These experiments have given a clear idea of the requirements for effective ICMS without tissue damage. A preliminary study of the histochemical composition of a number of Rhesus monkey forearm muscles has begun, utilizing animals that are sacrificed for other reasons. Cortical neuron function has also been explored using chronically-implanted microelectrodes in the sensorimotor cortex of cats during locomotion and postural activity. Of particular interest are: 1.) whether cortical cells located near one another behave similarly during a variety of normal, spontaneous movements as well as in response to sensory inputs; and 2.) whether sensory information to the cortex is gated during the performance of normal movements like walking. With respect to the first question, pyramidal tracts neurons sharing the same sensory input can behave quite differently during locomotion, depending on cortical location (and presumably, the destination of their axons to particular motor nuclei in the spinal cord). There is preliminary evidence for significant gating of sensory inflow to the motor cortex during the E₁ phase of stepping, or when the cat is lifted and dropped a short distance. These results require amplification and further controls before rigorous interpretation is possible but they illustrate the importance of exploring such questions in intact, behaving animals. Although the necessary experiments are complex and very time-consuming, the required information can be gathered in no other way.

Work under the project entitled "Models of Neural Interaction" has concerned the fundamental problem of pattern detection by ensembles of neurons and the more applied problem of extracting information about neural activity from multichannel data streams such as are obtained from intact, moving animals. One member of the Laboratory staff has begun a collaboration with the Department of Otolaryngology,

University of California, San Francisco, on a new model of pitch perception that is directly relevant to the development of a feasible auditory prosthesis for hearing-impaired patients. This model suggests that pitch information may be extracted by neurons in the medial superior olive that respond to very small differences in input timing arising because of mechanical traveling waves exciting relatively restricted regions of the cochlear basilar membrane. Testing of the model is being actively pursued by psychophysical experiments with human subjects and in acute animal experiments. Considerable effort is being expended to develop a computer-based data analysis system that will permit staff members to utilize the Laboratory's new computers to reduce the voluminous data streams that emerge from chronic implant experiments. In addition, the theoretical basis for extraction of neural activity information from multichannel neuroelectric recordings has been refined (Independent Source Theory) and, at the appropriate time, will be tested using firing patterns of alpha motoneurons and EMG recordings as the test system.

Work done under the project entitled "Techniques for Making Contact with the Nervous System" largely results from the needs and demands generated by other projects in LNLC, although some input is received from outside groups in terms of questions or specific fabrication needs. During FY 1980, a number of specific devices have been designed and fabricated, including multiple-lead cuff electrodes for implantation around peripheral nerve to permit conduction velocity measurements and a special-purpose timer/delay device for spike-triggered averaging. In addition, designs have been finalized for a modular series of signal processing devices that are adaptable for general use. Because of their flexibility and modular design, these instruments can be used in all of the experimental setups within the Laboratory, leading to considerable savings in equipment outlay and maintenance. Many of the methods developed in LNLC in the course of its own projects have been of considerable general interest and staff members identified with this project serve as sources of information for inquiries from around the world. An informal newsletter about matters relevant to biomaterials and chronic implantation of various devices has been circulated for several years and now reaches over 90 individuals regularly, at their request. Evaluation of the state of the art in biomaterials, electrode wires, insulations and electronic and electromechanical devices applicable to motor systems research and/or motor prosthesis applications continues constantly in LNLC and this information is made available to other groups at NIH and elsewhere.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01686-12 LNLC
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Motor Control Systems in the Spinal Cord		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Robert E. Burke, M.D. Other: Richard P. Dum, Ph.D. Robert F. Hutton, Ph.D. Michael J. O'Donovan, M.D., Ph.D. Martin J. Pinter, Ph.D. Aharony Lev Tov, Ph.D.	Chief Guest Worker Guest Worker Guest Worker Guest Worker Visiting Fellow	LNLC NINCDS LNLC NINCDS LNLC NINCDS LNLC NINCDS LNLC NINCDS LNLC NINCDS
COOPERATING UNITS (if any) None		
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SUMMARY OF WORK (200 words or less - underline keywords)		
<p> This project is designed to provide information on the mechanisms operating within <u>reflex</u> systems which include <u>alpha motoneurons</u> as the output link, as well as on the interconnection and interaction of different reflex systems. Particular consideration is also given to correlations between <u>synaptic organization</u>, intrinsic neuronal properties, and dynamic behavior of the <u>alpha motoneurons</u> and the <u>physiological</u> characteristics of the <u>muscle fibers</u> innervated by them. </p>		

Project Description:

Objectives: This project is designed to provide information about the organization of neuronal systems in the spinal cord of mammals which ultimately control the activity patterns of motor units (motoneurons and the muscle fibers they innervate), including the interaction between primary afferent and supraspinal descending systems in the control of information flow in spinal segmental motor mechanisms. Of particular interest is the organization of synaptic input systems that project to motor units of different types within a particular motor pool.

Methods Employed: A variety of experimental approaches have been used in this project but all are applied to study the lumbosacral spinal cord of the adult cat. Much of the work has been done on animals anesthetized with barbiturate, α -chloralose or inhalation (Halothane) anesthesia, or on unanesthetized animals following destruction of the supratentorial brain under rapidly-reversible inhalation anesthesia (decerebrate preparations). Most of such experiments have been devoted to intracellular recording from identified alpha motoneurons using conventional micropipette electrodes, with analysis of synaptic potentials produced by electrical stimulation of peripheral nerves and/or of selected supraspinal structures (e.g., red nucleus, reticular formation, motor cortex, etc.) using stereotaxically placed electrodes. In addition, we are using the method of intracellular iontophoresis of the tracer protein, horseradish peroxidase (HRP), to permit neuroanatomical study of functionally-identified neuronal elements in the spinal cord. Thus far, this method has been used mainly to study the intraspinal anatomy of identified muscle afferents (mainly group Ia fibers) in relation to their terminations on alpha motoneurons belonging to identified types of motor units. Within 8 to 15 hours after the first HRP injection, the animals are perfused transcardially with fixative under deep anesthesia, and spinal cords are then dissected, photographed and processed for demonstration of HRP (diaminobenzidine - cobalt method) in frozen section material.

Another series of experiments under this project is quite different, in that they involve study of the electrical and mechanical activity patterns of selected muscles in the cat hindlimb during normal motor activity (e.g., locomotion, jumping, posture, etc.) in intact cats that are essentially free to move. This work uses chronic implantation of recording devices such as electromyographic (EMG) electrodes, tendon force transducers developed in LNLC (see previous Annual Reports) and muscle length transducers, in conjunction with videotape recording of the animal's movements on a treadmill or overground (cf. report of Project Z01 NS 02080-07 LNLC, "Neuron Activity During Locomotion").

Major Findings:

A. Supraspinal Control of Cutaneous Excitation in Extensor Motoneurons.

Work on this subproject resumed in late FY 1979, with regard to the possible role of the rubrospinal and corticospinal tracts in the control of transmission of information in the interneuronal pathway carrying excitatory input from low threshold cutaneous afferents from distal hindlimb to ankle extensor motoneurons. This pathway is of interest because it tends to excite motoneurons of fast twitch (types FF and FR) motor units much more than the cells of slow twitch (type S) units. After considerable evaluation of alternative approaches, a satisfactory

experimental paradigm was developed to study this complex system in cats anesthetized with α -chloralose.

Intracellular recordings are obtained from ankle extensor motoneurons, identified as to muscle of destination by the pattern of group Ia excitation projecting to them. Synaptic potentials produced by electrical stimulation of the ipsilateral sural nerve from distal hindlimb skin are recorded with and without conditioning stimulation (short trains of pulses) delivered to the contralateral red nucleus or to the medullary pyramid via stereotaxically-placed monopolar electrodes. Test, conditioning and conditioning-plus-test trials are interleaved (minimizing distortion of the results due to drift of membrane potential, etc.) and separately averaged in a signal averaging computer controlled by an external logic network. A variety of controls are also used to determine that the supraspinal stimuli are delivered to the appropriate structures at appropriate strength (20 - 100 μ A pulses, 0.2 msec duration, trains of 4 - 10 pulses at 200 Hz), and that other, previously studied spinal pathways are appropriately affected (e.g., facilitation of disynaptic group Ia inhibition from antagonist muscle nerves). When all of these conditions are satisfied, there is a clear facilitation of sural nerve short-latency excitatory input to ankle extensor motoneurons from both rubrospinal and pyramidal stimulation. Detection of the rubrospinal effect on early excitatory components in the sural nerve response is complicated by concomitant facilitation of longer-latency inhibitory synaptic potentials (the IPSPs from the flexor reflex afferents, or FRA). The effect of pyramidal volleys is much more apparent on the short-latency excitation produced by low threshold (less than twice electrical threshold) sural nerve afferents because of less effective pyramidal facilitation of the later, higher threshold FRA IPSP components.

The demonstration of excitatory control by supraspinal systems of the excitatory interneuronal pathway between low threshold sural nerve afferents and ankle extensor motoneurons suggests that this segmental pathway, which we have previously shown to alter the functional threshold difference between fast and slow twitch motor units, can be controlled by motor commands from supraspinal centers that are generally thought to be involved in "voluntary" motor actions. This would provide an important degree of flexibility to the control of motor unit pools such that synchronous activation of an entire pool, or perhaps even preferential activation of its large force, fast twitch elements, would be possible during movements that require rapid force generation (e.g., jumping or ballistic limb movements) or that involve rapid alternation (e.g., paw shaking, scratching). Until recently, such possibilities have been regarded with considerable skepticism.

B. Anatomy of Muscle Afferent - Motoneuron Relations.

Experiments involving injection of the tracer protein horseradish peroxidase (HRP) into functionally identified group Ia afferents and into α -motoneurons postsynaptic to them (identified as to motor unit type) began in FY 1978 and have continued through FY 1980. Initial experiments were devoted to refining the methods for identifying neural elements and for injecting them with HRP. During FY 1980, the major problem has been to refine the method for reconstructing the complete trajectory of group Ia afferent collaterals, and of the complete dendritic tree of alpha motoneurons, from serial frozen sections. This has proven to be a formidable technical problem because of the complexity of these structures and their wide spatial extent. In large measure, the results obtained depend on the

plane of sectioning and the method for recording the position of HRP-labeled structures.

Our present approach is to cut the spinal cord containing four to six labeled motoneurons and a single labeled group Ia afferent in an unconventional oblique longitudinal plane that follows the plane of the Ia collateral arborizations, so as to limit the number of sections that contain elements of primary interest. Using this method, it is then practical to make transparent photomicrographic montages of each section on high contrast negative material at relatively high magnification, permitting overlay matching of structures in serial sections. This obviates the need for tracing highly complicated, intertwined structures under the camera lucida and permits reliable mapping and identification of every labeled element in each section. The "contacts" established by labeled afferents on labeled postsynaptic structures are defined in this work by examination under oil-immersion magnification. These contacts may or may not be functional synapses. Corroborative evidence can only be obtained by subjected each contact to ultrastructural study but this is unfortunately impractical at present. Our current view is that the contacts seen at the light microscope level represent maximum estimates for the number of functional synapses on any given motoneurons and that the actual number of these must be less than or equal to the observed number.

Our experience to date indicates that there may sometimes be considerable overlap between the territories of neighboring group Ia collateral arborizations, and that several collaterals can make synaptic contact with a given motoneuron, often on spatially separate regions. Multiple contacts on different dendrites appears to be the rule, and such contact regions also appear to occur at different electrotonic distances from the motoneuron soma. The vast majority of contacts have been found in the extensive motoneuron dendritic tree. A striking finding is that the two to three major collateral branches that form in the dorsal horn in any given Ia collateral can each have sub-branches that contact a given motoneuron, so that, if failure of action potential invasion into major different branches occurs (as has sometimes been postulated), the first branch points of consequence to a given motoneuron are those that form in the dorsal horn, one to two millimeters from the motoneuron. The available material is still too limited to give meaningful figures for the average number of group Ia synaptic contacts on individual motoneurons, or on the average density of Ia contacts in relation to postsynaptic motor unit type, but answers to these questions should come with an increased data base.

C. Recruitment Models for Motor Unit Populations.

Based on data about the organization of synaptic inputs to motor units of known type, it is possible to suggest "recruitment models" by which the output of a motor unit population can be predicted in terms of motor unit types active at particular levels of force generation. Testing such models requires methods for studying whole muscle and motor unit activity in intact, freely moving animals (cf. Project Z01 NS 02080 LNLc). Of particular relevance to the present project is the comparison of whole muscle electromyographic activity (EMG) and force output with the characteristics of the motor unit populations making up the same muscles. In this regard, previous work with the MG and soleus muscles has been extended to the flexor digitorum longus muscle (FDL). This muscle was chosen because of its relevance to nerve cross-union experiments reported in Project Z01 NS 02160 LNLc. Muscle force and length transducers have been adapted for the FDL in order to

characterize its mechanical activity during locomotion, jumping, etc., along with its EMG activity (as well as EMG in other limb muscles). Data obtained during FY 1980 show that the FDL is virtually silent during most of the stance phase of the step cycle at slow to moderate speeds of treadmill locomotion; almost all of the low force registered at the FDL tendon during stance can be shown to be passive. The main action of FDL comes in a short burst just after foot lift-off ("pre-flexion burst"), at which time little tendon force is recorded because the muscle is unloaded and is shortening rapidly. Somewhat greater FDL activity in stance occurs during fast running but FDL action is greatest during vertical jumps and in climbing, when the muscle can produce as much as 1 kg. in a short burst just at foot takeoff (the fully tetanized FDL can produce about 1.5 to 2.0 kg. isometric force). The FDL also exhibits irregular bursts of high activity during otherwise stereotyped locomotion, presumably to apply corrective forces needed to maintain balance or to adjust limb positions. The occurrence of such bursts cannot be predicted or controlled, and their lack of stereotypy make it difficult to analyze their functional "meaning". The primary action of FDL is to flex the distal phalanges and to protrude the claws, the latter action particularly important to the cat. The observations to date suggest that the FDL muscle is not a "prime mover" in locomotion but rather acts as an auxiliary muscle to correct inaccuracies of placement or perturbations of balance.

Another remarkable finding from this work is that the EMG activity pattern of the flexor hallucis longus (FHL) muscle is quite different from the FDL. FDL and FHL are close anatomical synergists since they both originate from common structures and their respective tendons join together before inserting on the distal phalanges. Moreover, FDL and FHL motoneurons are linked in close synergy with regard to group Ia muscle afferent interconnections. However, FHL exhibits strong stereotyped activity during the stance phase of locomotion (similar to the ankle extensors), in sharp contrast to the passive FDL which bursts instead at the onset of swing (see above). The finding of quite different dynamic activity patterns in two muscles that appear to be close synergists presents a serious challenge to accepted notions about the definition of muscular synergy. The central neural organizations underlying this difference remain to be clarified.

Significance to Biomedical Research and the Program of the Institute:

Active movement of mammals in space is accomplished by motor units with motoneurons located in the spinal cord. Analysis of the central nervous system control of movement requires a detailed understanding of the organization and interaction of input systems to the spinal cord segments, both from peripheral afferent sources and from supraspinal structures. There is now considerable evidence for the existence of functional specializations among the muscle fibers of different motor unit types, as well as among their α motoneurons. The long-range goal of the present project is to analyze the patterns of neuronal organization present in the spinal cord as they relate to the motor unit types in order to further our understanding of how motor units, and therefore movements, are controlled. The specialization evident in different motor unit types has long been thought to be associated with equivalent differences in functional usage. Definition of synaptic organization and direct study of motor unit usage patterns in normal movements are necessary to test this important hypothesis, as are direct studies of dynamic muscle usage in intact animals. Such studies are of clear relevance to analyses of both normal and abnormal movement patterns in man. They

also bear importantly on the interpretation of results of clinical investigations using electromyography and muscle histochemistry in normal human subjects and in patients with neurological diseases.

Proposed Course of the Project:

It is anticipated that present work on the interaction of primary afferent and descending control of transmission in the excitatory cutaneous pathway from distal skin to ankle extensor motoneurons will conclude in FY 1981 with study of the sites in sensorimotor cortex that produce the effect on the spinal pathway. Unfortunately, the relative weakness of descending effects, and their lack of precise timing (indicating polysynaptic transmission), make it appear unlikely that we will be able to utilize the descending systems to identify individual spinal interneurons as candidates for inclusion in the cutaneous excitatory pathway, as suggested in previous Annual Reports. During FY 1981, we will instead concentrate attention on enlarging our data base on the detailed anatomy of group Ia afferent collaterals, and on the anatomical structure of alpha motoneurons of identified motor unit type. A major unsolved question concerns the possibility that action potentials do not completely invade complex axonal arborizations like those of group Ia collaterals. Pilot experiments have begun in an attempt to analyze this question were directly, using electrophysiological recordings from Ia arborizations and from postsynaptic motoneurons during modulation of Ia transmission by post-tetanic potentiation. Major technical obstacles to the direct recording approach lie in the design of appropriate electrode arrays, and the very small amplitude of the electrical signals generated by the fine axons in Ia arbors. Work on the mechanical activity of FDL muscle was completed in FY 1980 but the problem of possible differences in synaptic organization between FDL and FHL motor nuclei, implied by their different usage patterns in locomotion, may be attacked in FY 1981, depending on progress in the other projects noted above.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 NS 01687-12 LNLC																																													
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SUMMARY OF WORK (200 words or less - underline keywords) This project is intended to develop techniques for the acquisition and processing of <u>neuroelectric signals</u> from the central and peripheral nervous system in <u>acute and chronic neurophysiological preparations</u> .																																															

Project Description:

Objectives: Successful monitoring of neural activity from the peripheral or central nervous system in intact, moving animals requires the development of novel techniques that apply to unique recording situations. The latter include recording from both acute and chronic preparations in which stable and discriminable single unit activity is required, but the problems are particularly critical in recording from animals that are awake, comfortable and either free to move or moving with minimal restraint. Delivery of functionally effective stimuli through metal micro-electrodes without damaging the electrode or causing pathological changes in nearby neurons has now become essential for ongoing laboratory experiments, and this requires considerations different from those involved in recording. This project is designed to evaluate methods, materials and designs to solve particular research problems in both recording and stimulation situations.

Methods Employed:

A. Evaluation of Materials suited for biological implantation.

The evaluation of the physical properties and biocompatibility of Parylene-C, metallic iridium and certain medical grade silastic rubbers has continued, primarily through examination of implanted materials for electrical and mechanical integrity, using electrical testing and/or scanning electron microscopy, and of site of implantation using routine histological methods.

B. Designs for chronic recording intracortical microelectrodes.

Fabrication techniques for making "map-pin" electrodes continue to be refined and modified, directed toward the production of "hair brush" electrodes in which two or more electrodes are combined so that the inner electrode tip spacing is 100 micrometers or less. An additional modification of the "map-pin" electrode includes a new design which is slightly larger and has a much blunter tip for recording from the ventral roots in the spinal cord of cats. The latter electrodes are mounted in a linear array of twelve such that the recording shafts extend about one centimeter from a silastic holder.

C. Nerve Cuff electrodes for recording from peripheral nerve in cat.

A specially designed electrode for recording from peripheral nerve in chronic preparations of cats has been perfected to include two sets of three electrode arrays. This configuration allows for convenient measurement of conduction velocity of motor and sensory nerves. The cuff electrodes are typically fabricated from molded silicone rubber with platinum-iridium wires embedded in them for recording or stimulation. Cuffs with fine tubes in them have been designed and used for injection of local anesthetic agents, to provide transient denervation of selected nerves, or even of selected groups of fibers within those nerves. Feasible dimensions for cuff electrodes on various nerves have been evaluated by in situ testing.

D. Implantable strain gauge for monitoring muscle tension from associated tendon.

The semiconductor tendon strain gauge developed in Laboratory of Neural Control several years ago continues to be implanted in cats as part of ongoing kinesiological studies. A recent modification of this device includes changes in relative geometric configurations in order to adapt it to large tendons such as the patellar ligament, and use of a two-arm bridge configuration to reduce temperature drift.

E. A new treadmill for kinesiological studies using cats.

A novel treadmill design has been developed to facilitate footfall detection, improve video optics, to allow tilting of the surface, to facilitate the study of reduced preparations (e.g. mesencephalic) and reduce electrical and acoustical noise. Construction is scheduled to begin in late FY 1980.

F. Pressure sensitive force plates for determination of weight distribution and center of gravity in standing cat.

This device (described in 1979 Annual Report) has been refined to test static dynamic forces of all four paws of a standing cat and has since been modified to include a photo device for detecting foot contact. Another device which has only one force plate has since been designed and built to detect forces in the X,Y and Z directions. This device has been used in studies of the flexor digitorus longus muscle.

G. Digitally controlled stimulator and isolator.

This system was described in the 1979 Annual Report but has since been refined. The stimulator, although digitally controlled, is not microprocessor based, due to speed requirements of on-line applications. The final instrument that was designed, built and is now in operation produces perfectly balanced biphasic waveforms in which the plus and minus phases are automatically kept equal for all pulse width and amplitude adjustments. A linear biphasic constant current/constant voltage isolator was also designed and built which interfaces the biphasic generator to the biological preparation.

H. Digital timer/delay instrument for spike triggered averaging.

An instrument has been developed which produces three independent pulse delays which are multiplexed onto one output to trigger a stimulus generating system. The pulse delays are typically triggered from a spike discriminator. Each pulse delay can be disabled and is assigned a voltage code which is multiplexed with the other pulse delays as well as the input trigger pulse for analog tape storage. The pulse code can then be played back off line and fed back into the same instrument, which then decodes the signal and the stimulus pulse delays can easily be synchronized to the recorded raw data. An LED readout is also provided to keep track of neural spike events. Other pulse codes are available for triggering a signal averager or external relays which switch a microelectrode connection from a record amplifier to a stimulus isolator. Other outputs are provided for various stimulus protocols for testing muscle dynamics.

I. Other laboratory instruments.

The following list includes some of the special-purpose instruments designed, built and put into operation in the laboratory within the last year: 1.) slope/level detector for amplitude and slope discrimination of analog signals; 2.) multi-channel differential amplifier system for interfacing the input transformer used for the nerve cuff electrodes; 3.) a high impedance differential amplifier for recording from bipolar metal microelectrodes; and 4.) a sample and hold circuit which is used to maintain a constant D.C. level output from an intracellular amplifier (i.e., to compensate for fluctuating membrane potentials) before the signal is fed to a tape recorder or signal averager.

Major Findings:

"Map-pin" electrodes continue to provide excellent success in recording chronically from single neural elements in various regions of the nervous system. Histological sections from cats that had arrays of three electrodes with less than 100 micron tip spacing ("hair brush" design) showed the same histological results as the single "map-pin" electrodes; i.e. no tissue reaction and evidently viable neurons within 100 microns of the electrodes.

Electrode implants that utilized the "hair brush" electrode design have successfully recorded from single neurons for over 110 days. It is interesting to note that individual electrodes that were part of a three electrode array (e.g., "hair brush") implanted in cat motor cortex recorded successfully for an even longer period of time.

A modified version of the "map-pin" electrode in which the recording tip was made blunter than normal, giving a relatively lower tip impedance, has replaced the fine wire technique previously used for recordings made from the ventral roots in intact cats. These electrodes have given much higher yields in recording from single nerve fibers as well as increasing the time over which one is able to record from an individual unit.

Ten nerve cuffs have been successfully implanted around the femoral nerve in cats over the past year for both recording and stimulation protocols. The size of unit potentials nominally ranged between 2 to 4 microvolts using spike triggered averaging. The length of time a nerve cuff was able to record distinguishable units was for two to three months.

The digital timer/delay instrument has been successfully utilized in several studies in sophisticated experimental protocols where spike triggered averaging of single motor units was sought. This study was done to confirm that a motor unit being microstimulated (to identify its twitch type) is the same as the motor neuron being chronically recorded from.

Significance to Biomedical Research and the Program of the Institute:

The successful development of techniques for recording signals from the nervous system, and for delivering safe current levels and waveform parameters for neural-stimulation, is essential to the success of ongoing experiments in LNLC. These newly developed techniques are also beneficial to other laboratories involved in neurophysiological research and ultimately may have an impact in the development of prosthetic devices for the neurologically handicapped.

Proposed Course of Project:

Continued use of the "map-pin" electrode design for recording neurons in the superficial layers of the cerebral cortex is anticipated. Appropriate modifications to the electrode designs will continue to be made in order to adapt them to other recording situations, with special emphasis on array designs and implant procedures for chronic spinal cord studies. Cats implanted with "map-pin" electrodes will be fitted with various EMG recording electrodes, strain and length gauges and nerve cuffs to permit detailed correlation of normal neural activity with various voluntary and reflex locomotory movement.

The new pressure sensitive force plate will be utilized in conjunction with chronically implanted cats fitted with various EMG, strain and length gauges, map-pin electrodes and wire electrodes to analyze normal neural activity with respect to postural and dynamic weight distribution as they relate to the activity of specific muscles and motor units.

Continued cooperation and communication with the Neural Prosthesis Program is anticipated. In addition, the information circular published annually by LNLC staff and supplied to investigators interested in chronic recording techniques will be continued. This informal publication now reaches about 100 investigators worldwide, at their request.

Publications

Chapin, J.K., Loeb, G.E. and Woodward, D.J. A simple technique for determination of footfall patterns of animals during treadmill locomotion. J. Neurosci. Meth. 2:97-102, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01688-12 LNLN																								
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SUMMARY OF WORK (200 words or less - underline keywords) <p> This project is designed to investigate the size and spatial distribution of cortical "colonies" that are associated with individual muscles or closely related groups of muscles, as well as the activity of neurons in such colonies in the motor cortex and <u>supplementary motor area</u> during defined voluntary motor behaviors. <u>Intracortical microstimulation (ICMS)</u> is used to map regions that produce excitation or inhibition of particular muscles or muscle groups, and the resultant cortical maps are compared with these for synergist or antagonist muscle groups. <u>Cortical cell discharge patterns</u> during normal movements is evaluated with respect to the excitation or inhibition of muscle activity that is produced by ICMS. A pilot study of the histochemistry of monkey forearm muscles has been initiated. </p>																										

Project Description:

Objectives: The major goals of this project are: 1) to examine the spatial organization of motor cortex outflow to particular muscles or muscle groups during free or goal directed movement in awake animals; 2) to determine whether the firing patterns of small sets of cortical neurons contain sufficient information to specify the details of motor performance; 3) to investigate the effect of afferent input to individual neurons in the sensori-motor cortex of cats during locomotion; 4) to examine the activity of closely located cortical neurons recorded from closely spaced electrodes to determine to what extent the sensori-motor cortex is organized into "colonies" of cells with similar function; 5) to determine if pyramidal tract (PT) neurons classed according to their sensory input respond in a similar manner under a variety of movements; 6) to determine the spatial distribution of fiber types in monkey forearm muscles with histochemical staining methods and compare these with muscle usage and cortical cell firing patterns during motor performance; and 7) to determine the safest, most effective stimulation parameters for intracortical microstimulation.

Methods Employed:

Monkeys are initially trained to produce defined movements of the wrist and forearm. In a current paradigm, the monkey manipulates a handle that positions a cursor light and is required to match the position of this cursor light to one of eight target lights for a prescribed length of time in order to receive a liquid reward. When the animal is well trained, a chamber to support the microelectrode drive is implanted over the hand-arm area of the motor cortex and a pyramidal tract stimulating electrode, EMG electrodes, and tendon strain gauge are implanted. After recovery from surgery, the monkey is retrained to move the handle between 3 pairs of targets under 4 different load conditions. Task correlated cells are sought, with a moveable microelectrode. After recording the task related parameters, EMGs, and the activity of a cortical neuron, microstimulation is applied through the electrode to determine which muscles respond. Sufficient cell firing data is collected to utilize spike triggered averaging to determine if the cell under investigation facilitates or inhibits the EMG activity of the implanted forearm muscles.

In cat experiments chronic "map-pin" electrodes are implanted in sensori-motor cortex along with a PT tract stimulating electrode, and nerve cuff recording electrodes. Microstimulation, through the cortical electrode is used to map muscles activated from specific cortical sites. Afferent input to identified PT neurons is evoked by physiological cutaneous stimulation or by electrical pulses delivered through implanted wires.

Studies of muscle histochemistry in monkeys have been performed on muscles removed from animals sacrificed in the normal course of other experiments. Forearm muscles are removed in toto and rapidly frozen in isopentane cooled with liquid nitrogen. 10 μ m frozen sections are cut from selected portions of the muscles and stained with alkaline and acid ATPase and NADH-tetrazolium reductase for identification of type I, type IIA and type IIB fibers.

Major Findings:

Intracortical microstimulation (ICMS) through a microelectrode while recording from chronically implanted EMG electrodes provides a means of determining how muscles are affected by stimulation of specific, highly-localized cortical sites. Both proximal and distal muscles can be activated by trains of pulses at current levels below 20 μ a. The effects in a given muscle can be generated by ICMS in widely separated areas, suggesting that cortical "colonies" related to a given muscle can be very extensive and perhaps multiple. Excitatory or inhibitory EMG responses to ICMS can occur within 11 ms after the start of the stimulus train and end 11 ms after the end of the train. The prompt termination after the end of the stimulus train suggests that the onset and offset latencies are due mainly to conduction and synaptic delays rather than to reverberating cortical circuits that might be expected to remain active after the end of stimulation. EMG patterns produced by ICMS have ranged from inhibition or excitation of a single muscle to simultaneous inhibition and excitation of synergists or antagonists. Areas that produce inhibition of a given muscle have been observed as close as 100 μ m to areas producing excitation of the same muscle. Cortical sites that produce inhibition in a single muscle tend to surround those areas where ICMS produces excitation in the same muscle. Pure inhibition of forearm muscles with ICMS has been observed predominantly in flexor muscles.

Task-related activity in individual cortical neurons has been evaluated at sites where either excitation or inhibition of forearm muscles was produced with ICMS. Cells that exhibited consistent alterations in firing patterns before and/or during specific muscle activity are referred to as correlated with the muscle(s). The majority of cells whose firing patterns were correlated with increased flexor muscle activity during the task were recorded at sites where ICMS produced flexor excitation. The majority of cells whose firing patterns were correlated with increased extensor muscle activity during the task were recorded at sites where ICMS produced extensor excitation and/or flexor inhibition. At sites where only flexor inhibition was produced by ICMS, the firing patterns of the majority of cells were correlated with extensor muscle activity. Cells whose firing patterns were correlated with flexor muscle activity could be involved in the excitation of flexor muscles and/or inhibition of extensor muscles. Likewise cells whose firing patterns were correlated with extensor muscle activity could be involved in excitation of extensor muscles and/or inhibition of flexor muscles. The fact that only flexor inhibition was produced with ICMS at specific sites where the majority of cells were correlated with extensor muscle activity suggests that these cells might be involved in active inhibition of flexor muscles. If some cortical cells produce only inhibition of muscle activity, then cells recorded at sites where ICMS produces inhibition should be active when the target muscle is inhibited. Likewise at sites where ICMS produces excitation, the cells recorded should be active when the target muscles are active. The majority of cells follow this expected ICMS pattern but a substantial number do not, suggesting that the cortical outflow to different muscles overlaps in space and that the functional effect of individual cortical cells may be complicated by their connectivity in the spinal cord segments to which they are assumed to project.

In order to minimize cell damage during ICMS, no net DC current should pass through stimulating electrodes. Minimizing the stimulating current required to produce a given functional effect will also help to reduce any possible tissue damage due to the stimulating current. The pulse width necessary to minimize the

charge required to stimulate neural tissue is in the range of 50 to 100 μ sec. A reduction in the charge necessary for functional effect can be achieved by using a balanced biphasic stimulating current, delaying the anodic repolarizing phase 100 to 200 μ s after the end of a 200 μ s cathodic stimulating phase. As the width of the stimulating pulse is increased, the charge reduction effect produced by delay of the anodic repolarization pulse is reduced.

The activity patterns of individual neurons in the forelimb region of the sensorimotor cortex of cats have been studied using chronic recording techniques during locomotion, postural adjustments and landing from falls. Attention has been directed primarily at those cells that project from the forearm area via the pyramidal tract (PT neurons). Almost all of the neurons so far recorded have shown modulation of their activity in relation to phases of the step cycle. As expected, a large proportion of these neurons have clearly circumscribed strong peripheral afferent input, mainly from the skin of the fore-paw. Movement-related modulation of PT neurons might be explained in terms of the varying barrage of afferent input generated by the movement. This aspect of the problem has been investigated by random electrical stimulation in the cutaneous receptive field of the units during walking. There are variations in the responsiveness of cortical cells to such volleys which appear related to phases of the movement and perhaps to the level of the EMG activity. However, these fluctuations in the sensory response do not operate to the same degree or at the same phases of the movement to account for the all of the modulation of the PT units during locomotion. In a few instances, intracortical microstimulation has been carried out via the cortical electrodes to examine the relationship between cortical activity and muscle activity. It has been found that the cortical microstimulation response does not appear to depend on the level of EMG activity. Thus, during a movement such as locomotion in the cat, there appear to be intervening relays between the pyramidal tract neurons and the alpha motoneurons which limit the correlation between cortical unit firing and muscle activity.

Another set of experiments in intact cats has examined the activity of single PT neurons during a variety of movements in which different levels of muscle force are required, and in which the same muscles are used in different combinations (e.g., comparing activity patterns during walking versus landing after the animal is dropped from different heights. Although a particular unit is modulated during both movements, the degree of modulation is less during dropping than would be expected if cortical activity were related to muscle force per se, independent of the type of movement. In dropping from heights, there is a large increase in EMG activity several times greater than any level seen during locomotion, and there is a co-contraction of the forelimb muscles not observed during locomotion. Thus cortical activity appears to be modulated in relation to the type of movement demanded rather than being related only to the level of activity in individual muscles.

In monkey forearm muscles studied thusfar, we have found a uniform mosaic of type I and type II fibers throughout the muscle in all but flexor carpi ulnaris (FCU). The fiber type proportions in most muscles are essentially constant across the muscle cross section. However, FCU is a bipennate muscle with distinctly different proportions of type I and type II fibers on either side of a central tendon. Brachioradialis in *Macaca mulatta* has a very low percentage of type I and type IIA fibers as compared to the other monkeys studied.

Proposed Course of Project:

Work will continue in the area of obtaining long-term chronic recordings from neurons in the motor-sensory cortex and supplementary motor area. Initially, studies will be conducted with a microelectrode controlled with a microdrive so that a large number of cells that are related to movement can be obtained from the supplementary motor area (SMA). This same technique will also be used in the motor cortex to map the areas that produce responses in selected arm muscles. Strain gauges will be implanted on the tendon of selected muscles to measure force with voluntary movements and with intracortical microstimulation. Muscle length will be measured by the position of the wrist while it moves a manipulandum. These measures, combined with chronic EMG recordings, will provide information on how specific muscles are utilized in a movement and on how specific cortical areas relate to these peripheral actions. The forearm muscles used are being studied histochemically and these data can be compared with usage of the muscles during the trained task. Data will also be correlated with cell firing patterns of neurons from cortical regions that produce either excitation or inhibition with ICMS. Pyramidal tract stimulation will be employed to identify neurons projecting into the pyramidal tract, the majority of which are corticospinal. To further delimit cortical projection to motor nuclei, spike-triggered averaging from cortical spike to EMG will be employed. The parameters of ICMS will also be investigated in these animals to further refine the most efficient and safe waveform for long-term intracortical stimulation. Chronically implanted intracortical capacitor stimulating electrodes will be evaluated for long term safety and stimulating effectiveness.

During the remainder of the fiscal year further tests will be conducted with cats to determine if the observed gating of ICMS occurs in the cortex or spinal cord. Force data from individual muscles will be obtained during the drop test to determine the pattern of muscle activation. These tests will provide sufficient information to allow termination of the cat experiments at the end of FY-80.

Significance to Biomedical Research and the Program of the Institute:

The motor cortex and possibly the supplementary motor area are intimately involved in the production of distal, exploratory movements with hands and digits in primates. These functions are disturbed by stroke in many human patients. The mechanisms of compensation for motor deficits caused by cerebral lesions are unknown but information about normal cortical mechanisms and their stability (or instability) with time is important to increase our basic understanding which then can be applied to lesion problems. Studies on the dynamic activity of cortical neurons and their relationship to movement along with the spatial organization of neurons related to a specific muscle or movement will provide this basic information. With our newer microstimulation and microelectrode recording techniques we can now better attack the question of whether the cortex contains a representation of muscles or of movements. Because all movements involve the contraction (or relaxation) of one or more muscles, the basic representation must be in terms of muscles but the large spatial extent of cortical "colonies" to a specific muscle and the overlap of the colonies that project to different muscles tend to indicate that any given cortical region may be involved in specifying combinations of muscles that produce a movement.

Utilizing EMG recordings along with intracortical microstimulation at specific times during a trained movement task allows evaluation of the efficacy of particu-

lar stimulation parameters. Comparisons can be made between intracortical metal and capacitor electrodes as to efficacy and long term safety. Determination of the safest stimulation values and electrodes will be directly applicable to neuro-prosthesis applications.

The ability of the chronically implanted "map-pin" electrodes to record the activity of the same cortical neurons for many days, weeks and even months should prove useful for evaluating drugs. This work may also have significance for the development of cortically-controlled prosthetic devices, although the electrodes are not yet satisfactory for obtaining prosthetic control signals where recordings for a number of years are required.

Publications:

Schmidt, E.M. and Thomas, J.S.: Motor unit recruitment order: Modification under volitional control. Prog. Clin. Neurophysiol. (in press).

Schmidt, E.M.: Single neuron recording from motor cortex as a possible source of signals for control of external devices. Annals of Biomed. Eng. (in press).

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SUMMARY OF WORK (200 words or less - underline keywords) <p> The overall objective of this project is to examine certain principles of organization within populations of neurons and to devise means of recording the activity of neurons in such populations. One such principle is that the independent components of the pattern of activity of a population of similar neurons are related to the physiological function of those neurons. Thus, among neurons of sensory systems, the stimulus features detected by the neurons at a given level would tend to be the independent components in the stimulus-generated pattern of input to them. One important type of feature would be detected by collecting correlated activities among the population of afferent neurons through delays and weights which would cause the components of the pattern to arrive simultaneously at a detector neuron. In motor systems, the independent components in the activity of a population of neurons would be an estimate of the degrees of freedom in the motor command signals and/or sensory information feeding onto them. Development of reliable methods for recording simultaneous activity of numbers of related neurons is necessary in order to test these models of organization. </p>																	

Project Description:

Objectives: This project continues to be concerned with pattern detection, both among neurons and as a tool for gathering data. It is now necessary to develop specific data processing programs to test the independent sources theory. At the same time computing tools are being developed for general use in the neurokinesiology laboratory. The primary concern in this project is with neuronal networks that detect patterns by convergent weights and delays, i.e., by temporal cross correlation.

A new collaborative study was initiated this year by Dr. Gerald E. Loeb with Dr. Michael Merzenich, Director of the Coleman Memorial Laboratory, Dept. of Otolaryngology, University of California San Francisco School of Medicine. The project concerns the elucidation of a neural network which could underlie the formation of a central representation of pitch on the basis of spectral pattern perception. The project is an outgrowth of Dr. Loeb's previous responsibilities as Project Officer on two extramural contracts for the development of multi-channel electrode arrays intended for use in a functional artificial ear. Recent theoretical and psychophysical research into the perception of pitch has pointed to the likelihood that pitch assignment by a subject listening to a complex sound is performed on the basis of some sort of "best fit" pattern extraction performed on an accurate CNS representation of the spectral and spatial cues present in the source. The two classical theories of acoustic spectral perception, cochlear place pitch and periodicity pitch, are now generally recognized to lead to serious limitations if they are, in fact, the only available cues for the system. In particular, the broadening of the tuning curves at high sound levels predicts a significant degradation of discrimination ability and changes in sound quality as intensity is varied over the wide dynamic range over which we know pitch perception to be nearly invariant. This project seeks to develop a model of the brain stem processing of auditory signals which would account for both these normal psychophysical data and the percepts elicited in human subjects by intracochlear electrical stimulation as employed in current auditory prosthesis projects.

Methods Employed

During the last year, LNLC replaced its obsolete PDP-12 computers with the smaller, cheaper and more powerful PDP-11's. After considerable evaluation of alternatives, we have adopted the programming language 'C', a third generation computer language developed at Bell Laboratories on the PDP-11, as the basic language for LNLC systems. The high level interactive language APL is also being adapted for use in circumstances where programs must be modified frequently and where relatively slow operating speed can be tolerated.

For the auditory project, metal microelectrode recording techniques are being used to record single cell activity in the cat anteroventral cochlear nucleus (ACVN) and medial superior olive during acoustic stimulation and electrical microstimulation in AVCN. Intracochlear multi-electrode arrays similar to those employed in human auditory prosthesis implants are also used to study the precise timing of response to various electrical waveforms.

Major Findings

A number of data analysis programs of increasing difficulty have been written on the PDP-11 computer for general laboratory use. These are of immediate benefit to the entire staff of LNLC and, in addition, lead toward programs designed to test the theory of independent sources outlined in earlier annual reports. The theory has been generalized so that it is better adapted to the problems of our laboratory. The analysis programs and the new form of the theory are outlined below.

The data analysis programs are being written for and used primarily in the neurokinesiology laboratory where we intend to assemble a comprehensive system for processing and displaying multichannel data (EMG, unit, length, force, etc.), at first off-line but eventually with capacity to write and use programs quickly during experiments. Programs written since September include routines designed as follows:

- 1) To recognize the swing phase of stepping in the signal from a length gauge implanted in the forelimb of walking cats.
- 2) To use the times of these swings to align spike and EMG traces in rasters, one line for each step.
- 3) To accumulate these rasters in histograms, as representative steps.
- 4) To accumulate data over long periods of time from moving animals into pulse height histograms, where the heights indicate levels of activity.
- 5) To compute the mechanical power and energy output of muscles from length and force signals.
- 6) To fit points to arbitrary mathematical models by using the program MLAB which is implemented on the NIH DEC-10. Data is accumulated on the laboratory computer and transmitted to the computing center by a phone link.

A. Locomotion Results

The data analysis programs developed in this work have been used in FY 80 mainly to assist with reduction of data streams from experiments on locomotion in the intact cat, particularly in the work involving recording from individual neurons in the sensorimotor cortex during stepping, postural perturbations and landing from falls (see Project Z01 NS 01688-12 LNLC). Data from 17 PT units of the foreleg area have thus far been analysed in detail by rastering with respect to the time of foot fall, footlift and/or the transition between flexion and extension. Activity patterns in all of the cells studied were modulated in correlation with the step cycle. The PT units seemed to be grouped into two classes, those that showed increased firing rates closely linked to particular points in the flexion or extension parts of the swing phase, and those that showed a decrease in activity during either part or all of swing and the beginning of stance. Units located near to one another were usually members of one of these two classes, and also sometimes shared other step-modulation characteristics. For some units, superimposed on a regular modulation there were fluctuations which varied from step to step. In some of these, a variable burst during the flexion phase became visible when the rasters were summed. This seemed to occur when extra force was required to stabilize the step cycle. These results illustrate the problems faced in separating stereotyped activity from superimposed fluctuations of uncertain origin in neuronal discharge patterns. Given sufficient attention to the characteristics of the movements performed, it is anticipated that much of the non-stereotyped "fluctuations" will be found to correlate with particular aspects of movements that are not precisely

repeated. Dealing with this problem is a major challenge to current work on movement control in intact, behaving animals.

B. Independent Source Theory

The theory, described in previous Annual Reports, has been extended to allow recovery of signals of independent sources from mixtures of these signals even when the links between sources and recording points are frequency dependent. This allows for cases in which the medium introduces delays and distortions, so long as they are linear. Thus if X^λ is the λ frequency component of the vector array of sources X , and Y^λ is that for the array Y of recorded signals, then

$$Y^\lambda = A^\lambda X^\lambda + N^\lambda ,$$

where the matrix A^λ contains the linkages: the ij element of A^λ is the contribution of source j to the recording point i at frequency λ . N^λ is noise. We can solve for A^λ from the correlations among the Y^λ as follows:

$$A^\lambda = U^\lambda \text{FACT}(C^\lambda) ,$$

where C^λ is the correlation matrix of Y^λ : $C^\lambda = E(Y^\lambda Y^{-\lambda})$, and E stands for "expectation of". "FACT" denotes the factor analysis algorithm. U^λ is an array of unknown coefficients that cannot be derived from pairwise correlations such as C . However, one can find it from higher order correlations. For example if the sources have non-zero third moments ("spikey sources"), then

$$U^\lambda = \text{EIG}(E(Y^\lambda Y^{-2\lambda} Y^\lambda))$$

where EIG denotes an eigenvector procedure, which in this case is applied to the third cross-moment array of the data Y^λ . This gives A^λ , from which one can easily derive the coefficients of a multichannel filter for extracting the time courses of the sources from the recorded signals. Left out of this outline are calculations of the number of samples that would be required to achieve a required accuracy, and a modification of the method appropriate for sources whose third moment is zero.

C. Auditory project results

A new theory has been devised which states that pitch might be extracted by detecting small time differences in discharge between cochlear nerve fibers which result from the sequential activation of cochlear nerve fibers as the mechanical traveling wave produced by a sound moves down the basilar membrane. The theory suggests that a range of pitch sensations could arise from single short segments of the basilar membrane which would account for some puzzling psychophysical observations which have been made on human subjects who have been fitted with stimulation electrodes in the scala tympani as part of the auditory prosthesis project.

The collaborative project with UCSF involves the elucidation, in animal experiments, of the neural pathway which could subserve the fine temporal cross-correlation function required by the theory. The present emphasis is on the cells of the medial superior olive, a structure already known to be able to detect fine inter-aural timing delays (on the order of 20 μsec) and which is well situated in

the ascending auditory relay chain. The theory is being refined to generate reasonably precise predictions of the neuronal activity which might be anticipated at the various stages of the processor; preliminary neurophysiological experiments have been carried out to confirm some of these predictions and demonstrate the feasibility of an experimental demonstration of the theory. We have demonstrated that the "primary-like" neurons of the anteroventral cochlear nucleus respond to electrical stimulation in the scala tympani with phase locking to 6.4 kHz and inter-fiber temporal dispersions of 100-200 μ sec., both characteristics shared by these units during normal acoustical stimulation.

Significance to Biomedical Research and the Program of the Institute:

The independent sources algorithm may provide a basis for the prediction of the general form of feature-detecting networks from an analysis of the data they analyse. For example, a model of part of the auditory system could be made by analysing the signals from two microphones in the presence of a sound source and interfering sources. The weights and delays generated as appropriate for optimally converging the received signals onto one detector neuron would constitute a hypothesis for a neuronal detection system for that sensory problem. Similar suggestions were made in a previous Annual Report for predicting the optimal mixtures of receptor signals in color vision, and for finding the independent degrees of freedom in the coordinated use of muscles. The algorithm also suggests detection systems for laboratory problems such as sorting of multichannel EMG signals. Thus, the theory of independent sources can provide a practical basis for solving difficult data analysis problems encountered in many types of neurophysiological research, in addition to its value in generating experimental hypotheses.

The auditory work seems important not only for what it may contribute to a more comprehensive understanding of sound perception, but also for its relevance to the practical problem of developing a successful auditory prosthesis. Efforts are presently underway at a number of institutions around the world (including UCSF) to develop a functional auditory prosthesis which would permit unaided speech perception by the totally deaf. It has become increasingly apparent that the basic principles of pitch perception on which previous designs have been based are not adequate to account for the clinical experience to date with prototype devices. The collaborative project on auditory encoding is specifically designed to test alternate hypotheses of both normal and prosthetic pitch perception, in order to develop a new theoretical framework upon which future development of the artificial ear can proceed in an intelligent and orderly manner.

Proposed Course of Project

The next year will be spent mainly assembling and writing additional programs for the neurokinesiology laboratory and in implementing and testing the independent sources algorithm. In previous annual reports, tests of simpler independent sources models without distortion or delay were described. These had to be tested with simulated data because most real data include both of these complexities. To test the present model we may use a physical model system, such as three independent audio sources and about five microphones as recording points, each receiving delayed, distorted mixtures of the source signals. Noise can be added by including additional low level sources, unique to each pickup or correlated between

them. The sources can be rectified to introduce third moments, or the waveforms can be left symmetrical about the mean. Analysis of this test will require the use of the fast fourier transform, complex factor analysis, and complex eigenvector algorithms, instrumented in the laboratory computer. A biological test system has been discussed in previous Annual Reports - an attempt to obtain multiple single unit EMG signals from multichannel, multiple unit records. Human or animal EMG data can be used, obtained by introducing a needle into a muscle carrying a number of adjacent fine recording leads. The major problem is expected to be keeping the number of high amplitude units small compared with the number of leads that record different mixtures.

A series of increasingly flexible programs for gathering data for general use is planned. These will include programs to log multichannel events which occur both before and after an event marker such as a stimulus pulse. More sophisticated displays with easily placed axes and labels will also be needed. To enable unsophisticated users to gather data, manipulate it, and display it, a high level language, probably BASIC or perhaps APL, will be augmented to provide flexible data acquisition and display.

The presently begun series of electrophysiological studies in the brainstem auditory nuclei of anesthetized cats will be pursued vigorously to test several specific predictions of the theory. Human subjects who may become available through the auditory prosthesis project will be tested psychophysically with whatever tests are suitable for the electrode configurations and other hardware available.

Publications.

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02080-07 LNLCL																																																		
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TITLE OF PROJECT (80 characters or less) Neuron Activity During Locomotion																																																				
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SUMMARY OF WORK (200 words or less - underline keywords) A variety of new techniques are being used to monitor the <u>afferent</u> and <u>efferent neural activity</u> in the <u>spinal cord</u> and <u>motor cortex</u> of intact cats during normal and perturbed <u>locomotion</u> . Flexible wire electrodes in the cerebral cortex lumbar <u>dorsal root ganglia</u> (DRG) and <u>ventral roots</u> record stable, identifiable unit activity which is correlated with kinesiological data from chronically implanted gauges of muscle force, length, and EMG activity developed for this project. Neurons are characterized by conduction velocity, anatomical origin, and modality using <u>spike-triggered averaging</u> of EMG signals and neurograms obtained from specially designed <u>nerve cuff electrodes</u> implanted around peripheral nerves. The <u>reflex effects</u> of various <u>electrical stimuli</u> to motor and cutaneous nerves are systematically examined as they vary through the step cycle.																																																				

Project Description:

Objectives: The major goal of this project is to examine directly the roles of spinal neurons and primary afferent fibers in normal movement which up till now have only been inferred from paralyzed or decerebrate acute preparations. The principal current emphasis is on natural patterns of afferent and efferent activity in the spinal cord and the role of afferents as part of a system of servo-mechanisms. Spinal reflexes elicited by both cutaneous and proprioceptive afferent activity are now known to have a profound effect on locomotory patterns and the effect of such perturbations on movement and neural activity are also being studied.

Methods Employed:

1. The present method for obtaining stable afferent unit records during normal walking consists of inserting insulated 50 μ m diameter wires into the dorsal root ganglion (DRG) via a small laminotomy. The cut ends of the wire constitute the recording surface and the only fixation is by a flexible Silastic carrying sleeve sutured to the dorsal spinous process.

2. A similar technique has been successfully employed in the ventral roots of the 5th lumbar spinal segment. Efferent unit activity in the axons of motor neurons is identified as such by spike-triggered averaging of the records obtained from a cuff electrode chronically implanted around the femoral nerve and from EMG electrodes designed to sample each of the five anterior thigh muscles to which this nerve projects (further description in the report of project Z01 NS 01687-12 LNLCL).

3. Supporting kinesiological measurements include continuous read-out of positional signals at ankle, knee, and hip joints via implanted length gauges, of the force generated by individual muscles via chronically implanted tendon strain gauges, and of overall movement patterns by videotape gait analysis.

4. Techniques have been developed to implant large numbers of bipolar recording and stimulating electrodes at a variety of sites in both hindlimbs of a freely walking cat, permitting analysis of reflexes elicitable by electrical stimulation of various afferent classes during stepping.

5. A new technique has been developed for microstimulating motor axons being recorded through the same microelectrodes. Under favorable conditions, it is now possible to characterize the muscle fiber types innervated by the recorded motor axon by recording the tension profiles produced by microstimulation with an implanted strain gauge. In practice, we electronically link such stimulation to the time of natural spike occurrence, using the refractory period of the muscle fibers to confirm that the recorded and microstimulated units are the same ("spike-triggered microstimulation").

6. A new technique has been developed which allows the selective infusion of nerve blocking agents such as xylocaine around a given peripheral nerve while the animal is behaving normally. Since axons of differing size can be selectively and reversibly blocked pharmacologically, it is in principle possible to examine the effects of sudden losses of afferent information or fusimotor control on the activity of the remaining neurons.

7. The technique of generating controlled decerebrate locomotion by stimulating the midbrain locomotory region has been successfully combined with recordings from single spinal units previously observed during normal locomotion.

8. An extensive hardware and software system has been devised to facilitate interactive reduction and analysis of neurophysiological and kinesiological data produced by the various projects using the neurokinesiology facility.

Major Findings:

Work to date in this and other laboratories regarding the normally occurring activity of muscle spindle afferents has demonstrated a number of very different patterns, each of which would tend to support only some of the conflicting theories of muscle spindle function. The central question is whether these afferents are, in fact, accurate sensors of muscle length and/or velocity of length changes, or whether their complex and powerful intrafusal fibers are used dynamically to create afferent patterns which subserve some other role (e.g. trajectory error detection). One possibility is that the variety of experimental observations may be attributed to the different muscles and tasks which different investigators have chosen to study. This would then imply that spindles do subserve multiple roles, a situation consistent with the high degree of intrafusal control arising from the gamma (and possibly beta) motoneurons, a prominent feature of most mammalian motor neuron pools.

Our study of single motoneuron and spindle afferent activity from the bi-articular and bifunctional anterior thigh muscles, rectus femoris (RF) and sartorius (SA), has revealed an interesting dual organization which may reflect on the general principles of motor control employed in locomotion. Previous kinesiological studies here and elsewhere have indicated that the flexor and extensor muscles have mechanically different tasks to perform during normal locomotion, the flexors generally shorten in a rapid, stereotyped manner during swing while the extensors generally undergo lengthening or isometric contractions as they support the body weight in a spring-like manner. The RF and SA (pars lateralis) muscles are knee extensors and hip flexors and generate two EMG bursts, one during extension (stance phase) and the other during flexion (swing phase). It now appears that different motor units contribute to each phase of activity and that units recruited by one burst are not recruited during the other even if the EMG recorded from the whole muscle is considerably larger in the burst where the unit is inactive. This is surprising in view of previous work regarding the orderly recruitment of successive motor units as level of effort in each muscle increases, a concept known as the "size principle." This dual motor organization appears to be reflected in the gamma (intrafusal) as well as alpha (extrafusal) motoneurons. Spindle afferents have been recorded which are active in proportion to the muscle length and velocity of stretch. Their behavior suggests little gamma static bias to these spindle afferents even during alpha activity. This behavior is typical of spindles we and others have recorded from pure extensor muscles. Other spindles fire briskly during the rapid, active shortening of the swing phase, suggesting strong alpha-gamma (static) coactivation, a property which we and others have attributed to pure flexor muscle spindles. In both groups, spindle activity is maintained during the active use of the muscle (or functional sub-grouping), but the physical correlates of the activity and, hence, its significance for regulatory feedback, are very different.

The data base on motoneuron discharge patterns during normal locomotion is slowly expanding, with results not appreciably different from those described in previous Annual Reports. In particular, the discharge frequencies of individual motor units in cat hindlimb muscles exhibit much wider ranges than had been

supposed on the basis of experiments in anesthetized animals. Because units can be studied completely only under unusually favorable conditions, the data collection process is slow but the method remains the only one practical for obtaining the required information in intact animals.

Significance to Biomedical Research and the Program of the Institute:

Much current work on the study of mammalian locomotion has been concentrated on the cat hindlimb, where considerable knowledge is already available concerning the physiological and anatomical properties of the muscles, motor neurons, afferents, and spinal reflexes. However, the details of the functioning of this system during normal locomotion under cerebral control can at present only be inferred, giving rise to a number of competing control theory hypotheses. The new methods employed in this project should provide data needed for testing such hypotheses and formulating new ones. An understanding of the normal control of movement is essential to understanding a number of degenerative diseases of the spinal cord (e.g. ALS, transverse myelitis, etc.) that affect locomotion. A longer range application of the techniques using chronic transducers and afferent monitoring is in the field of functional neuromuscular stimulation (FNS). Sophisticated devices designed to restore motor function by bypassing CNS lesions (e.g. in paraplegics) will probably require some form of closed loop servo-control utilizing transducers of muscle length and tension and of skin pressure. If it proves possible to obtain stable afferent activity rather than using implanted artificial transducer signals over long periods of time, afferent recording electrodes could improve the function and simplify the design and implantation of complete FNS systems.

Proposed Course of Project:

A major objective of this project is the determination of the functional spinal organization underlying the generation and regulation of locomotion. The most promising sources of data appear to be the single unit activity of muscle afferents (particularly spindle endings), the EMG and force outputs of selected muscles, and unitary activity from identified spinal cord motoneurons. The experimental paradigms to be investigated include the various "voluntary" modes of locomotion, the effects of singular perturbations such as electrical and mechanical stimuli during locomotion, and the effects of various deprivations such as deafferentation, local anesthesia, mechanical and neurological deafferentations, and loss of environmental (e.g. visual) cues. This work will continue in order to enlarge the present data base. Improvements in methods are constantly being sought but the basic framework of this effort is now solidly established.

A long-term technological goal is to determine the limits of recording device stability and tissue compatibility in both time and numbers of information channels with a view to assessing the feasibility of deriving somatosensory information for the feedback control of Functional Neuro-muscular Stimulation Prostheses.

Publications:

Duysens, J. and Loeb, G.E. Modulation of ipsi- and contralateral reflex responses in unrestrained walking cats. J. Neurophysiol. (in press).

Duysens, J., Loeb, G.E., and Weston, B.J. Crossed flexor reflex responses and their reversal in freely walking cats. Brain Res. (in press).

Hoffer, J.A., Loeb, G.E. Implantable electrical and mechanical interfaces with nerve and muscle. Annals Biomed. Engr. (in press).

Loeb, G.E. Somatosensory unit input to the spinal cord during normal walking. Canad. J. Physiol. and Pharm. (in press).

Loeb, G.E. and Hoffer, J.A. Muscle spindle function during normal and perturbed locomotion in cats. Exp. Brain Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 NS 02160-06 LNLC																																													
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PI:	Robert E. Burke, M.D.	Chief	LNLC	NINCDS																																											
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SUMMARY OF WORK (200 words or less - underline keywords) This project is designed to provide information on the ranges and distributions of the electrophysiological and morphological characteristics of <u>alpha motoneurons</u> and of the interrelated mechanical, histochemical and morphological properties of the <u>muscle fibers</u> innervated by them (i.e., the muscle unit). The <u>motor unit</u> populations in normal animals are compared with those in animals after various conditioning treatments.																																															

Project Description:

Objective: This project is designed to provide information about the populations of motor units that make up large limb muscle in mammals, including the electrophysiological and morphological characteristics of spinal cord motoneurons in relation to the mechanical, histochemical and anatomical properties of the muscle fibers (termed "muscle units") innervated by them. The neural and muscular elements are functionally inseparable, making the motor unit the quantum element in all motor behavior. The collection of detailed data about the characteristics of normal motor unit populations permit assessment of alterations that are produced by various experimental manipulations, including changes in mechanical demand or central nervous system lesions, or after axonal damage and reinnervation by foreign motoneurons.

Methods Employed: For the most part, analysis of motor unit properties is carried out in anesthetized cats using methods of intracellular recording and stimulation of individual spinal motoneurons to ensure functional isolation of individual motor units. Electrophysiological properties intrinsic to the motoneuron, and the quantitative and qualitative characteristics of synaptic inputs to the cell, are evaluated with conventional techniques. The mechanical properties of the muscle unit innervated by each cell is then assessed during stimulation of the motoneuron through the intracellular pipette electrode, with the innervated muscle attached to a force-measuring device under isometric conditions. Muscle fibers of individual motor units can be labeled by depleting intrafiber glycogen during prolonged stimulation of the motoneuron, permitting histochemical and morphological study of the muscle units belonging to physiologically-characterized individual units. We have recently evaluated an additional method for muscle unit labeling using autoradiography after intervenous injection of ^{14}C -2-deoxyglucose during repetitive stimulation of individual motor units. Classification of motor units by type is done using methods and criteria described in earlier Annual Reports. Analysis of the numbers and spatial organization of motoneurons innervating particular muscles has been done using intramuscular injection of the tracer protein, horseradish peroxidase, to label cells in the target motor nucleus by retrograde transport.

Major Findings:

A. Properties of Muscles and Motor Units after Self-reinnervation or Cross-reinnervation by Foreign Motoneurons.

It is widely accepted that alpha motoneurons regulate in great detail the characteristics of the muscle fibers innervated by them but the mechanisms for this regulation are much debated. Strong evidence for such regulation comes from observations on muscles that have been reinnervated after damage to the normal motor axon supply. We have examined this model by characterizing motor units in the flexor digitorum longus (FDL) or soleus (SOL) muscles 9 - 13 months after their nerves had been completely severed and rejoined surgically. Self-reinnervated motor units in the FDL muscle can be classified into the same categories as found in normal FDL; the major difference from normal is that the reinnervated units exhibit a wider than normal range in tetanic tension output, implying a wider than normal range in the number of muscle fibers innervated per unit. In self-

reinnervated SOL, all studied units were type S, as in the normal muscle. The mosaic of histochemical muscle fiber types, which we have shown to be matched exactly with the different categories of motor unit types, is disordered in reinnervated FDL muscles, but there are no new or abnormal fiber types present. Identification of individual muscle units by glycogen-depletion and ^{14}C -2-deoxyglucose autoradiography shows that, while the association between physiological and histochemical fiber types is normal, the spatial distribution of muscle unit fibers is abnormal after reinnervation. All of these findings together imply that an unknown but probably substantial fraction of muscle fibers must convert completely from one physiological/histochemical type to another after self-reinnervation, under control of the new innervating motoneuron. We have begun a pilot mathematical mapping study of normal and self-reinnervated histochemical mosaics to assess whether it is possible to determine statistically how many fibers must convert from one type to another in order to produce the altered histochemical maps.

Another model that has been used to evaluate the "trophic" influence of motoneurons on muscle fibers is that of cross-innervation of nominally "fast-twitch" muscles by motoneurons of normally "slow-twitch" pools, and *vice versa*. A project to evaluate the effects of cross-innervation on individual motor units was begun in FY 1978 and data collection was largely completed during FY 1980. The data base includes whole muscle mechanical properties, wet weights and histochemical fiber compositions, as well as samples of individual motor units from cross-reinnervated FDL and SOL studied physiologically (motoneuron properties, synaptic input patterns, axonal conduction velocities, muscle unit twitch and tetanic responses, and fatigue resistance). A number of individual units have also been identified for histochemical analysis by glycogen-depletion of unit fibers. These data from altered muscles/motor units can be compared with equivalent data from normal and self-reinnervated muscles/units from this Laboratory (described above and in previous Annual Reports). A major advantage of the intracellular approach to motor unit isolation is that the innervating motoneuron can in each case be identified with assurance as belonging to either the FDL or SOL motor nucleus by virtue of its position within the spinal cord and the pattern of group Ia synaptic input received, which remains unchanged by cross-innervation. Self- and cross-reinnervated muscles are being stored frozen in liquid nitrogen for further analysis, which includes study by Dr. G. F. Gauthier (Univ. of Massachusetts Medical School) with immunocytochemical methods for the various subfragments of fast and slow muscle myosins. As a last stage, the residual blocks of the same material will be subjected to gel electrophoresis to provide quantitative data on their overall myosin composition.

Initial results of the cross-reinnervation study were described in the Annual Report for FY 1979. Cross-reinnervation of FDL muscle by SOL motoneurons (SOL \rightarrow FDL) produces virtually complete conversion of the normally heterogeneous FDL into pure slow twitch (histochemical type I) muscle, with marked resistance to fatigue. The 32 individual SOL \rightarrow FDL motor units studied were all type S and the 3 successfully studied by glycogen-depletion had type I muscle fibers. The normal and self-reinnervated FDL contain only about 10 percent type S motor units and about 11 percent type I muscle fibers, suggesting strongly that the vast majority of previously fast twitch fibers/muscle units are converted to type S/type I by reinnervation with SOL motoneurons. SOL \rightarrow FDL motor units frequently exhibit post-tetanic depression of twitch responses, as found in normal SOL units but very uncommon among normal FDL type S units. An important point is that cross-

reinnervated FDL muscles are much smaller than normal in wet weight (mean = 0.51g; normal mean = 1.01g; self-innervated mean = 1.0g). The individual type I fibers in SOL→FDL muscles are also considerably smaller than the overall average (mean fiber area 1323 μm^2 versus 1684 μm^2 for normal and 1895 μm^2 for self-reinnervated FDL). However, the mean for SOL→FDL fibers (all type I) is slightly greater than that for normal and self-reinnervated FDL type I fibers alone (1022 and 1149 μm^2 , respectively). In two cases, the cross-innervated FDL was extremely small (< 0.3g) although physiological testing showed that these muscles were in fact successfully reinnervated. The reason for this apparent atrophy and for the variation between individual animals is unknown but dynamic studies of cross-innervated muscles (see below) suggests that an imbalance between metabolic demand and available blood supply may be an important factor.

In contrast to the above results, cross-innervation (FDL→SOL) of the normally homogeneous SOL muscle (virtually 100 percent type I fibers and type S motor units) by the heterogeneous FDL motoneuron population (about 90 percent fast twitch motor units) produces muscles that are normal in size, wet weight and gross appearance, and fiber areas are similar to normal and self-reinnervated SOL. Remarkably, all of the FDL→SOL muscles studied histochemically exhibit virtually normal histochemical characteristics (all greater than 90 percent type I fibers), and most would be difficult to distinguish from normal SOL without other information. However, FDL→SOL muscles had whole muscle twitch contraction times about half (mean 61 msec) those found for normal (mean 140 msec) or self-innervated SOL (mean 119 msec). Three cases of dual innervation by FDL and some SOL motor axons was found, in which fibers innervated by the foreign FDL axons had an aggregate twitch contraction time of 61 msec while those self-innervated by SOL axons had a contraction time of 108 msec under the same mechanical conditions. These muscles also showed no histochemical mosaic in stains for myosin properties at any pH, but fibers innervated by the FDL axons had much less neutral fat content than SOL-innervated fibers. All 23 FDL→SOL motor units studied were typical type S, with characteristics essentially the same as normal SOL units, even though all were definitely innervated by FDL motoneurons. However, their twitch contraction times were faster (mean 53 msec) than normal (mean 97 msec) and fewer units showed post-tetanic depression than normal (1/16 tested versus 10/26 in normal SOL).

Both cross-innervations studied in this work (FDL → SOL and SOL → FDL) produce slow twitch (type S) motor units as identified by criteria developed for normal populations, although the heterogeneous FDL motoneurons do produce muscle units with faster contraction times than in SOL muscle fibers. There is evident dissociation between observed contraction speed and myosin histochemistry in FDL→SOL motor units which remains to be evaluated by immunofluorescence histochemistry and by gel electrophoresis of myosin subfragments. It seems quite possible that cross-reinnervation produces changes in the sarcoplasmic reticulum that result in the observed speeding of FDL→SOL twitch contraction times. Such changes would not be apparent in the routine histochemical stains used to evaluate these muscles.

B. Dynamic Characteristics of Self- and Cross-Reinnervated FDL and SOL Muscles.

The dynamic usage patterns of self- and cross-reinnervated muscles have never before been studied during normal movement in intact animals. Methods developed in this Laboratory (see previous Annual Reports of Projects Z01 NS 01687 LNL and Z01

NS 02080 LNLc) permit monitoring of chronic electromyographic (EMG) activity and of muscle force and length changes in freely moving cats. These methods were applied to 4 cats with self- and/or cross-reinnervated FDL and SOL muscles, and the results were compared to data from normal SOL (see previous Annual Reports) and normal FDL (see report of Project Z01 NS 01686 LNLc). The major finding was that self- and cross-reinnervated muscles behave dynamically exactly as expected for the innervating pool of motoneurons. There was no evidence at all for changes in dynamic activity patterns as a result of cross-innervation, including responses during treadmill locomotion, jumping, climbing, scratching and foot shaking. Reflex responses of the cross-innervated muscles during electrical stimulation of the sural nerve in the intact, moving animals were also unchanged from those found in normal cats. This lack of central reorganization after cross-reinnervation confirms previous, less complete data in the existing literature and fits with the evident lack of alterations in group Ia EPSP patterns found in the present work.

The cross-reinnervated FDL muscle (SOL→FDL) is active throughout the stance phase of locomotion and during all postural activity, as well as during running and jumping. This pattern, characteristic of the normal SOL motor pool, represents a major increase in FDL dynamic duty cycle, since the normal (and self-reinnervated) FDL is active only in short bursts, mainly during the early swing phase of locomotion. In contrast, the cross-reinnervated SOL (FDL→SOL) behaves like the normal FDL, with short bursting activity and little or no action during stance or postural maintenance. Thus, the duty cycle of the FDL→SOL muscles is very much reduced from normal. It seems quite possible that the major factor that induces the observed changes in cross-reinnervated FDL muscles is this very large increase in its functional duty cycle. It also seems possible that the occurrence of some SOL→FDL muscles with truly atrophic fibers and small wet weights (< 0.3g) may be due to idiosyncratic limitation of major blood vessels feeding into those muscles, which might be insufficient to deliver the blood flow necessary to sustain frequently repeated total recruitment of the SOL→FDL motor unit population, which is the case with the normal SOL motor pool. Experimental testing of this suggestion seems beyond the current state of the art. The much less dramatic changes found in FDL→SOL muscles (and motor units), however, challenges the notion to duty cycle or activity pattern is the major determinant of muscle fiber type. The SOL muscle fibers retain their essential type S character, including very marked resistance to mechanical fatigue, despite a very much reduced duty cycle.

C. Evaluation of ¹⁴C-2-deoxyglucose Autoradiography as a Marker for Active Muscle Fibers in Individual Muscle Units.

For more than a decade, depletion of intrafiber glycogen has been used in this and other laboratories as a marker to identify the muscle fibers innervated by a single motoneuron stimulated repetitively for prolonged periods. This method is not completely satisfactory for identification of fatigue-resistant fiber types that are very resistant to depletion of their already low glycogen levels, or in abnormal muscles (e.g., after cross-innervation) where glycogen levels can be quite low in many fibers. We have thus been evaluating an alternative method for fiber labeling, using ¹⁴C-2-deoxyglucose (2-DG) autoradiography as developed for metabolic studies of brain nuclei by Dr. Louis Sokoloff and his group in NIMH. In collaboration with Dr. Carolyn Smith of Dr. Sokoloff's group, we have injected 2-DG intravenously while stimulating an individual motoneuron with repetitive trains. After 30 - 90 minutes of stimulation, the target muscle is removed, frozen in toto

and later processed by cryostat sectioning to obtain serial sections for conventional glycogen staining (PAS method) and other histochemical reactions, and for autoradiography by several different techniques. Considerable effort has been expended to determine the optimum conditions for autoradiography in order to maximize cellular resolution, while minimizing artifacts such as chemography (which has proven to be severe in muscle, and dependent on muscle fiber type). The method has now been shown to produce excellent cellular resolution under the proper conditions. Autoradiographic maps of active muscle unit fibers fit well with glycogen-depletion maps and indicate the presence of additional active fibers not recognizably depleted of glycogen. Further studies are now possible that can determine quantitatively glucose utilization by resting and active fibers of different types, and of fiber type usage during normal activity at different levels of effort.

D. Histochemical Composition of Cat Leg Muscles.

The projects described under number Z01 NS 02080 (Neuron Activity During Locomotion) are now directed importantly at study of the knee extensor and hip flexor muscles in the quadriceps femoris group, and of the sartorius. These muscles have not been studied in any detail for intramuscular anatomy and histochemical fiber composition. Therefore, we have undertaken such a study during FY 1980. Analysis of this material will be completed in FY 1981, but it is already apparent that these muscles have highly complex internal architecture and significant regional variations in muscle fiber type composition. These points are extremely important to analysis of EMG and single motor unit studies now in progress.

E. Review of Motor Unit Populations and Their Relation to Movement Control.

During FY 1980, Dr. Burke completed a large scale review of motor unit physiology, histochemistry, morphology and functional correlations for a forthcoming volume in the Handbook of Physiology series, by invitation of the American Physiological Society.

Significance to Biomedical Research and the Program of the Institute:

Analysis of the control of movement by the central nervous system requires consideration of the properties and functional specialization of motor units, since they are the quantal elements from which all skeletal movements are composed. Studies of the interrelation between the intrinsic properties of motor units, including both the motoneuron and muscle unit portions, and the organization of synaptic input to the same units, have aided our understanding of the control problem and have suggested new avenues for research. In addition, elucidation of the detailed interrelation between the physiological, morphological and histochemical characteristics of muscle units in animal muscle has immediate relevance to investigations of human neuromuscular disease, in which electromyography and muscle histochemistry play important diagnostic and research roles. There is growing evidence that the basic pattern of motor unit organization in animals and man is similar in principle. In particular, the histochemistry of limb muscles in the cat and in man is remarkably similar. Studies of the effects on motor unit populations

of altered usage, CNS lesions, and denervation - reinnervation in the cat have important relations to the interpretation of clinical investigations in patients with neuromuscular disorders, peripheral neuropathies and CNS lesions. This is particularly true of current studies of the effect of foreign reinnervation on well-characterized motor unit populations.

Proposed Course of the Project:

Experiments on self- and cross-reinnervation between the FDL and SOL muscles have been completed but a final evaluation of muscle histochemistry will continue into FY 1981. Collaborative work on this material using immunofluorescent histochemistry of myosin subfragments, with Dr. G. F. Gauthier, has begun and will continue through the same period. When these studies are complete, the remaining muscle blocks will be sent to the laboratory of Dr. Susan Lowey, of Brandeis University, for biochemical analysis of myosin subfragments in normal, self-reinnervated and cross-reinnervated muscles. We are hopeful that all of this work will be complete by the end of FY 1981. Data analysis of the dynamic activity patterns of self- and cross-reinnervated muscles will continue into FY 1981 from tape-recorded material. Evaluation of the 2-DG method within LNLC will be finished by the end of FY 1980 but will continue at the University of Maryland, with possible continued collaboration of LNLC.

Publications

Burke, R. E. Motor units in mammalian muscle. In: Sumner, A. J. (Ed.) The Physiology of Peripheral Nerve Disease. New York: W. B. Saunders (in press).

Burke, R. E. Motor units: Anatomy, physiology and functional organization. In: Brooks, V. B. (Volume Ed.) Handbook of Physiology. Section 1. The Nervous System. Vol. 4. Motor Systems. American Physiological Society. (in press).

Burke, R.E. The stability of motor unit types in response to altered functional demand: Hypertrophy, atrophy and reinnervation models. In: Proc. of XXVIII International Congress of Physiological Sciences. (in press).

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neurophysiology
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neurophysiology

National Institute of Neurological and Communicative Disorders and Stroke

Henry G. Wagner, M.D., Acting Chief

For convenience, the research of the laboratory will be described according to several work objectives. One of the objectives has been to gain insight into the nature and function of the receptors in neuronal membranes. The principle question we have focussed on is, how do certain endogenous and exogenous ligands alter or modify the excitability of the neurone? We have utilized mammalian CNS neurons grown in tissue culture as our preparation. The cultured cells are dissociated from embryonic mouse spinal cord. Some experiments have used a clone of prolactin secreting pituitary cells. Both cell types have proven useful for pharmacological study of the receptors in the membrane. Intracellular electrophysiological recordings are made on individually selected cells to determine the characteristics of the cell membrane responses which have been induced by the various endogenous substances and exogenous drugs. We use double microelectrodes which permit voltage clamping the membrane and measurement of conductance changes in the membrane current. The naturally occurring endogenous substances and drugs are applied to individual cells by iontophoresis or pressure.

Virtually all spinal cord cells respond to each of the naturally occurring neutral amino acids. Individual cells exhibit a non-uniform distribution of responsiveness over the membrane surface suggesting that there is clustering of receptor sites for these substances. The elementary electrical events underlying the chloride ion (Cl^-) conductance change induced by the different analogues, and estimated Cl^- conductances and durations caused by these substances have been studied. Each of the amino acid responses is associated with a unique set of electrical properties. The estimated durations are significantly correlated with biochemical data on the same analogue.

All cultured spinal cord cells respond to pharmacological applications of L-glutamate. As with the neutral amino acids, responsive sites seem to be clustered. Our analysis of the elementary electrical activity associated with this substance revealed that a minority can be described within the framework of ion-channel behavior similar to that observed with the neutral amino acid responses. Further studies are planned.

We have applied the opioid peptides leucine, peptide substance P, and methionine-enkephalin to cultured spinal cord cells. A minority of the cells studied thus far responded to the peptides. When responses are present, they are (1) actions like the amino-acid transmitter, or (2) show a modulation of the transmitter-like response or (3) alter the threshold for action potential generation. Somewhat similar responses were seen with GABA and clinically important drugs as well. We have also

applied purines and pyrimidines to cultured spinal cord neurones and find transmitter-like effects. As with the above substance, a minority of the culture cells tested appear to be sensitive to these substances.

The effects of several classes of clinically important drugs on cultured spinal cord neurones have also been studied. We have found that the three classes of anticonvulsants (benzodiazepines, barbiturates and hydantoins) potentiate inhibitory responses evoked by GABA and reversibly attenuate the amplitude and duration of paroxysms in cultured cells which have been rendered "convulsant" by treatment with certain convulsants such as picrotoxin.

We have begun to study a clone line of pituitary cells which secrete prolactin. The majority of these cells are electrically excitable. Application of nanomolar concentrations of the tripeptide thyrotropin releasing factor induces complex and long lasting changes in the membrane properties of the cell. "Fluctuation" analysis indicates that the peptide induces channel like mechanisms similar to that involved in neurotransmitter actions on spinal neurones.

One of the major problems of cultured neurones is the considerable variability of the results obtained. These differences are not well understood. Cytochemical methods are being devised to identify neurons using antibodies for a number of cytoplasmic antigens. We hope to be able to stain particular neurones in the vital state revealing specific receptor sites so electrophysiological measurements can be made uniformly. Another possible determinant of variability may be in the growth media. We are experimenting with a variety of nutrient additives to improve or correct this problem.

Another work objective has been to correlate function and morphology in identifiable neurones in a complex ordered sensory pathway such as the visual system. The retina is a highly ordered neural plexus in the pathway which contains not only the sensory transducer mechanism but also a number of interacting neurones which can be reached individually for electrophysiological recording and staining. Single cell marking techniques are used to identify the cell. We first make an electrophysiological identification and characterization, then, inject horseradish-peroxidase (HRP) into the cell, and do a histological search for the cell and its dendritic extensions. Particular attention has been given to the horizontal cells and cones of the salamander. These cells are among the largest of vertebrate retinal cells. This factor may be the explanation for the relative ease of impaling and holding such cells for an adequate time to make careful study. We have known for some time that retinal cones can be depolarized by illumination of surrounding receptors. This effect is presumed to be mediated through the horizontal cells. We have now observed that intracellular injection of chloride ions into salamander cones enhances the depolarizing effect of the surround illumination on the cones. Extrinsic current injection which depolarizes the cone reverses the polarity of "the surround effect". We conclude from this

finding that the "surround effect" is due to a synaptically mediated increase in chloride conductance of the cone cell membrane.

Attention has also been given to the amacrine, bipolar and ganglion cells of the turtle retina. At least 20 different varieties of amacrine types alone have been attributed to this retina based on morphological characteristics. We uncovered evidence that about one third of the ganglion cells appear to have only bipolar input. They have color opponent center-surround spatial antagonism and stratify their dendrites more or less into a single layer of the innerplexiform layer. The remaining two thirds of the ganglion cells show both bipolar and amacrine input with little evidence of color opponency. Examination of HRP injected cells shows diffuse stratification of their dendrites in the innerplexiform layer.

Investigation was made of spectral and spatial coding in the receptive fields of the retinal ganglion cells of carp. The retina of fish are among the most complex cellular organizations of all retinae. Our purpose was to examine more carefully some difficulties with the conventional determinants of receptive field size. The two determinants most used are the sensitivity profile and the area-summation (Ricco) plot. For the center of the receptive field of a particular ganglion cell, these two measurements do occasionally agree but often serious differences exist which cannot be dismissed. We have collected a large number of instances where the data is confusing and also where it agrees. Since fish retina show spatial color opponency, an exceptional opportunity has been present to obtain this kind of data. Our analysis has opened up a number of avenues which should clarify this discrepancy and provide better questions to pose to this retina and lead to a better understanding of the nature and organization of this fundamental property.

Collaborative research continues to be very active between scientists of this laboratory and the scientists of other laboratories and institutes. Within the NIH, particular mention will be made of close collaborative efforts with scientists of IDB (NINCDS) NEI, NIMH, NIA. In the local area, mention will be made of Georgetown University and Duke University. More distant collaborations include scientists of the Institute National de la Sante, Recherche Medical, Bordeaux, France; Stanford University; University of California in Los Angeles; Laboratorio di Neurofisiologia del C.N.R, Italy; University of Newcastle on the Tyne England; University of Montreal, Canada; University of Texas in San Antonio; School of Victorian Pharmacy, Australia; Merck Frost Laboratories, Canada; and the University of Pittsburgh, Pittsburgh, Pa.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02019-08 LNP
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Electrophysiology and Neuropharmacology of Simple Cellular Systems		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: OTHER:	T.G. Smith J.L. Barker R. Canada K. Futamachi R. Study B. Dufy D. Mathers W. Vaughn W. Sheriff J. Mazzetta K. Saunders	Section Chief Medical Officer Staff Fellow Staff Fellow Staff Fellow Guest Worker Visiting Fellow Computer Specialist Computer Specialist Technician Technician LNP, NINCDS LNP, NINCDS LNP, NINCDS LNP, NINCDS LNP, NINCDS LNP, NINCDS RSB, NIMH ICS, IRP, NINCDS LNP, INCDS LNP, INCDS
COOPERATING UNITS (if any) Research Services Branch, NIMH; R.N. McBurney, University of Newcastle School of Medicine, England; ICS, ODIR, IRP, NINCDS		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Sensory Physiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.5	PROFESSIONAL: 3	OTHER: 1.5
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SUMMARY OF WORK (200 words or less - underline keywords) Electrophysiological experiments using intracellular recording techniques and extracellular pharmacologic applications have been performed on <u>mouse spinal neurons</u> and <u>pituitary cells</u> grown in tissue culture and on <u>molluscan central neurons</u> . The research has focussed on the mechanisms or how endogenous and exogenous ligands alter <u>neuronal excitability</u> . We have found that <u>endogenous amino acids</u> , <u>peptides</u> , <u>purines</u> and <u>pyrimidines</u> and <u>exogenous benzodiazepines</u> and <u>barbiturates</u> can all produce transmitter-like effects on cultured neurons. We have also found that certain pharmacological agents can have direct effects on non-synaptic membranes. We have applied "fluctuation" analysis to the transmitter-like actions and find that both endogenous and exogenous agents act in a similar manner to open ion channels. We have begun to characterize other forms of chemical excitability revealed by pharmacological applications of these substances. One insight we plan to pursue is the notion that the pharmacologic actions of some drugs may be mediated through receptors for endogenous ligands.		

Project Description

Objectives: Characterization of chemical forms of excitability on excitable membranes and the modes of action of clinically important drugs in regulating excitability.

Methods Employed: Intracellular recordings are made from individual neurons using conventional and voltage clamp techniques. Endogenous substances and exogenous drugs are applied locally to the surface of individual cells using iontophoretic and pressure methods. Electrophysiological assays of membrane events induced by various substances can thus be made relatively easily with each cell serving as its own control. The accessibility of the neurons growing in monolayer culture and ganglia dissects from the molluscan central nervous system permits long-term stable intracellular recordings which are critical for producing accurate measurements of membrane excitability. Experiments are designed to study membrane events with one of three levels of analysis: 1) single electrode membrane potential and conductance measurements; 2) double electrode voltage-clamp analysis of membrane current events and 3) fluctuation or "noise" analysis of membrane current fluctuations. The initial observations are made at the first level of analysis. These are followed up in more detail using the other techniques. Fluctuation analysis is a statistical treatment of membrane current fluctuations which occur as a result of the simultaneous opening and closing of many agonist-activated ion channels. The analysis is performed off-line with a digital computer.

Major Findings: 1. Neutral amino acid pharmacology. All spinal cord cells grown in culture respond to each of the neutral amino acids glycine, β -alanine and γ -aminobutyric acid (GABA). Cultured neurons show a non-uniform distribution of responses to the amino acids suggesting the development of "hot spots" or clusters of receptors on the cell surface. The most commonly studied amino acid response is functionally inhibitory to cell excitability and consists of an increase in Cl^- -conductance. We have studied the elementary events underlying the increase in Cl^- ion conductance using the technique of fluctuation (or "noise") analysis. The technique has previously been applied to chemical excitability at neuromuscular junctions where it has been used to estimate the properties of the elementary events underlying the excitability. Fluctuation analysis consists of a statistical treatment of the minute electrical fluctuations generated by the pharmacological application of transmitter substance. The estimates of the elementary events derived from the statistical treatment are in close agreement with direct measurements of the events themselves, thus providing support for the validity of the statistically derived values. We have used fluctuation analysis to estimate the electrical dimensions of elementary events associated with membrane current responses to a variety of structural GABA analogues. The results show that the average conductance of a channel activated by an analogue is not significantly different from that activated by GABA while the durations of analogue-activated channels are all significantly different from that

of GABA. Glycine-activated channels are approximately twice the conductance and one-quarter the lifetime of those activated by GABA. Although we have not completed a detailed study of comparative potencies of the different analogues, preliminary results show good agreement between the effectiveness of an analogue in evoking macroscopic current responses and the duration of the macroscopic conductance event.

We have correlated the data on channel duration with previously published values on the potency of the same GABA analogues in displacing labelled GABA from receptor sites on frozen rat and human brain membranes. The correlation coefficients are equal to or greater than 0.90 with binding data from six independent studies. All of these correlations are significant at the .01 level. The results suggest that the biochemical and electrophysiological assays measure some parameter(s) common to GABA receptor function. It is thus possible that the analogues are agonists at GABA receptor sites similar to those used in " Na^+ -independent" binding studies. The present results do not yet allow us to specify exactly how the binding of the agonist to the GABA receptor determines the duration of the conductance change, since neither the biophysical nor binding experiments distinguish between binding and conformational equilibria.

Less than 10 percent of cultured cells derived from dorsal root ganglia responded to GABA with a Cl^- -dependent depolarization. These cells do not respond to either glycine, β -alanine (or glutamate). Fluctuation analysis of the GABA responses reveals single channel estimates similar to those recorded on spinal cord cells.

2. Acidic amino acid pharmacology. All cultured spinal cord cells were excited by pharmacologic application of the acidic amino acid L-glutamate. The excitatory responses to glutamate are distributed in a non-uniform manner over the surface of individual cells. Fluctuation analysis of the current responses shows that less than 20 percent of the responses appear to be interpretable in terms of an elementary event resembling those underlying the neutral amino acid responses. Those that can be interpreted are associated with channels of less than 5 msec average duration and 10 pica-semens (pS) average conductance. We have begun to examine the effects of structural analogues of glutamate in order to determine whether there are different types of mechanisms underlying superficially similar excitatory responses.

3. Analysis of spontaneously occurring membrane events.

Fluctuation analysis of the majority of spinal cord cells recorded in the presence of tetrodotoxin and elevated magnesium ions (to block synaptic activity) and in the absence of exogenously applied substances shows that resting membrane properties can be described by a relatively simple "1/f" spectrum. Such a spectrum has been observed on other excitable membranes and is thought to reflect the summed activities of ionic conductance mechanisms associated with the resting membrane. These simple spectra are present over the -40 to -100 mV range of membrane potential. A minority of cells possess either discrete synaptic-like current events

and/or fluctuations in baseline membrane current. Fluctuation analysis of the baseline activity reveals spectra characteristic of those observed with chemical excitability. The discrete baseline events may reflect quantal synaptic currents, while the other activities associated with baseline recordings may represent a form of tonic transmitter release.

We have also studied the baseline activities of cultured sensory neurons derived from the dorsal root ganglion. About 50 percent of these cells have electrically quiet membranes devoid of any discrete events or membrane current fluctuations. On the remaining half we have observed both discrete voltage events and fluctuations at the resting membrane potential. These spontaneously occurring activities are always hyperpolarizing in direction and are insensitive to tetrodotoxin. They disappear abruptly as the membrane potential is hyperpolarized and cannot be reversed in polarity. Superficially the hyperpolarizations resemble synaptic potentials and are dependent on the presence of Ca^{++} ions. Under voltage clamp the potentials are replaced by outward-going discrete current events and fluctuations in membrane current. These current activities disappear as the membrane potential is hyperpolarized and cannot be reversed in polarity.

Amplitude and interval histograms of the voltage and current activities reveal the presence of elementary-sized events which are not precisely distributed in either a Gaussian or Poisson manner. The apparent amplitude of the elementary current event is 40pA at -45mV, giving an estimated elementary conductance of about 1.3nS. This is about 400-fold greater than amino acid-activated Cl^- ion channel events. All of the discrete events and fluctuations are blocked in a reversible manner by local application of 5mM tetraethylammonium ions, which blocks K^+ conductances on a variety of excitable membranes. There is as yet no evidence that the activity is sensitive to any drugs known to modify amino acid or acetylcholine responses. Spectral analysis of these events shows that the fluctuations are well fit by a single Lorentzian equation similar to that used to describe chemical excitability at synapses. From the cut-off frequency of the Lorentzian equation we estimate the average duration of an event to be about 10 msec. The present data do not reveal the origin of the events. Morphological studies at the light and electron-microscopic levels should provide some necessary evidence to distinguish between either an endogenous or exogenous source.

We have begun to study naturally occurring membrane events on GH3/6 cells, a clone line of pituitary cells which secrete prolactin. The majority of cells studied thus far are electrically excitable. A small percentage of these cells spontaneously generate action potentials and a minority possess spontaneous fluctuations of the baseline membrane potential. Spectral analysis of membrane current at the resting potential shows that the majority of cells have a simple $1/f$ spectrum while a minority possess spectra which appear to be better fit by a Lorentzian equation.

4. Peptide Pharmacology. The opioid peptides leucine and methionine-enkephalin have been applied locally by microiontophoresis and by pressure, and perfused in the bathing medium. Three functionally distinct types of membrane events have been observed: 1) transmitter-like responses, including a rapidly depolarizing, rapidly desensitizing excitatory response and an inhibitory response whose null potential is similar to that of GABA and is thus likely due to an activation of Cl^- conductance; 2) modulation of amino acid transmitter-like responses independent of any other effects on membrane properties; and 3) alteration in threshold for action potential generation. All of these pharmacological effects of the opioid peptides can be blocked by bath application of naloxone, suggesting that they involve stereospecific engagement of opioid peptide receptors on these cells. Entirely similar membrane responses except the threshold effect have been seen with iontophoresis and pressure application of the undecapeptide substance P. Spinal cord cells responding to either the opioid peptides or substance P numbered less than 20 percent of those tested with contrasts with the ubiquitous amino acid responsivity. A major goal of future research on peptide pharmacology with this preparation will be to enrich the cultures for cells expressing functional peptide receptors.

5. Purine and Pyrimidine Pharmacology. Although purines and pyrimidines are important constituents of nucleic acids, they are also released from neural tissue in a Ca^{++} -dependent manner and thus may mediate certain forms of intercellular communication in the nervous system. In addition, the purines hypoxanthine and inosine displace ^3H -Benzodiazepines in binding assays, leading to the suggestion that they may be endogenous ligands for benzodiazepine receptor sites. We have applied purines and pyrimidines to cultured spinal neurons and find two major, transmitter-like effects: a rapidly desensitizing depolarizing excitation similar to that described with peptide applications and a non-desensitizing inhibitory response due to an increase in Cl^- conductance. Less than 20 percent of the cells tested appeared to be sensitive to either type of endogenous substance. Clear interactions between the purines, pyrimidines, peptides and amino acids have not yet been demonstrated. The results provide pharmacologic evidence that these substances could mediate several forms of synaptic transmission in the central nervous system.

Nanomolar amounts of the tripeptide thyrotropin releasing factor cause the release of prolactin from pituitary cells in vivo and from the GH3/6 clone cells. We have begun to examine the effects of nanomolar concentrations of TRH on the electrical excitability of GH3/6 cells. TRH induces a short-lasting hyperpolarization of the resting membrane potential and a longlasting increase in fluctuations of membrane potential. Initial spectral analysis of the underlying current fluctuations reveals that they can be fit by a single Lorentzian equation, suggesting that like chemical excitability at synapses TRH effects on pituitary cells involve activation of channel-like mechanisms. The ionic determinants of these channels remain to be determined.

6. H^+ ion effects. H^+ ions applied by iontophoresis from pipettes containing HCl (pH-4) mimicked several of the membrane effects observed with iontophoretic and pressure applications of peptides, purines and pyrimidines including 1) a rapidly desensitizing, depolarizing excitatory response, 2) antagonism of neutral and acidic amino acid responses apparently in a competitive manner, and 3) elevation of threshold for spike generation. The results may relate to rapid titration of protein groups associated with specific membrane functions (e.g., rapidly desensitizing excitatory conductance mechanisms, amino acid receptor proteins, and surface charge relevant to activation of voltage-dependent conductances). They have implications for all observations made using iontophoresis in vivo. The latter research may be contaminated by contributions of H^+ ions, especially in those instances where the pH of the drug solution in the iontophoretic pipette has been lowered so as to positively charge the drug molecules.

7. Molluscan Neuron Physiology and Pharmacology. A newly identified group of neurons in molluscan ganglia, which normally display only randomly firing infrequent spikes, can be made epileptogenic by several procedures. These include replacement of extracellular calcium and strontium and the addition of certain drugs (e.g., picrotoxin). The epileptogenic activity is similar to that seen invertebrate central nervous system neurons and is characterized by randomly occurring, large depolarizing shifts in membrane potential and a rapid burst of spikes. Under voltage clamp, the untreated neuron shows an all-positive current-voltage curve. On the other hand, the epileptogenic neuron has a region of negative slope and is N-shaped. In addition, antiepileptic drugs (e.g., the hydantoins) return to normal electrical activity and remove the region of negative slope. Analysis of these changes shows that they are the membrane basis for the neuron becoming epileptogenic and the mode of actions of the antiepileptic drugs. Thus while synaptic activity and loci play an important role in the development of seizures and in the action of antiepileptic drugs (see below), these experiments suggest that non-synaptic membrane mechanisms may play an important and fundamental role in epileptogenesis.

8. Pharmacology of Clinically Important Drugs. Several classes of clinically important drugs have been applied to cultured spinal neurons. Bath application of agents which induce convulsions in vivo including picrotoxin, pentylentetrazole, penicillin, bicuculline, and strychnine cause paroxysmal depolarizing events in tissue cultured neurons. These events involve intermittent 20-30mV depolarizations which elicit high frequency repetitive spike firing. Under voltage clamp the depolarizations appear to consist of many discrete inward-going synaptic-like current events which invariably invert in polarity between -20 and 0mV. The depolarizing events are dependent on extracellular Ca^{++} and blocked by tetrodotoxin. We are presently doing fluctuation analysis of the synaptic-like events to see if it can be described by a single Lorentzian equation and whether the events evoked by different convulsants have similar properties at this level of membrane assay.

We have found that three classes of anticonvulsant including benzodiazepines, barbiturates and hydantoins are able to attenuate the amplitude and duration of the paroxysmal events in a reversible manner. The anticonvulsant activities are associated either with an elevation in the threshold for spike generation or a depression in the ability of a cell to fire repetitively. Local application of convulsants and anticonvulsants to individual cells reveals that five different types of convulsants including picrotoxin, bicuculline, penicillin, pentylenetetrazole, and strychnine all lower the threshold for single and repetitive spike while the three classes of anticonvulsant elevated threshold for single and repetitive spike firing. Preliminary results show that the anticonvulsants can reverse the effects of the convulsants on electrical excitability on the same cell. These results suggest that regulation of electrical excitability is an important site of drug action. Presumably the changes in threshold would contribute to the clinical effects of the drugs.

We have also found that lowering of threshold for single and repetitive spike firing can occur following local application of nanomolar concentrations of GABA on about 20 percent of the cells studied. We have found a good correlation among those cells responding to bicuculline and those responding to GABA suggesting that bicuculline may actually be a GABA agonist at this site. Our next step will be to complete some of the phenomenological details before proceeding to study the underlying mechanisms.

A second site of action of clinically important drugs is associated with modulation of amino acid-mediated Cl^- ion conductance. The convulsants depress conductance responses evoked by GABA and glycine. Fluctuation analysis of these interactions shows that while picrotoxin, bicuculline and strychnine depress amino acid responses they do not change the electrical dimensions of single channels activated by amino acids. Pentylenetetrazole and penicillin appear to shorten the average duration of GABA-activated channels without altering their average conductance. The anticonvulsant phenobarbital and the anesthetic pentobarbital both appear to increase the average duration of GABA-activated channels without altering their average conductance. Whether these observations at the microscopic level can account for the modulatory effects seen at the macroscopic level will require more detailed analysis.

The results with picrotoxin, bicuculline and strychnine on single channel properties may be interpreted as indicating that those channels contributing to the depressed response are operating in a manner indistinguishable from control, as if the drugs are able to eliminate a proportion of the amino acid-activated events in an all-or-none manner. Alternatively, the drugs may act in a fashion which cannot be resolved by fluctuation analysis. Competitive binding assays have shown that bicuculline and strychnine can displace GABA and glycine from receptor sites respectively while picrotoxin has little, if any effect. The results from fluctuation analysis coupled with the evidence derived from binding

assays suggests that bicuculline and strychnine may act at the level of the amino acid receptor while picrotoxin does not appear to act at this level.

The results with the barbiturates may be interpreted with reference to the observations made with amino acid analogues which shows that the relative potency of an analogue in displacing GABA from synaptic membrane sites correlates well with the "molecular efficacy" of the analogue. The barbiturates might thus increase the molecular efficacy of GABA by enhancing the affinity of GABA receptor sites for the amino acid. Recent evidence from binding assays shows that barbiturates can indeed increase the apparent affinity of GABA for its receptor, consistent with the above interpretation of the results from fluctuation analysis.

We have also found that the stereoisomers of the anesthetic pentobarbital can produce transmitter-like effects on cultured spinal neurons. The (+) isomer produces a predominantly excitatory action over the low-to-moderate range of concentrations. At higher concentrations the isomer indirectly inhibits excitability by increasing membrane conductance, presumably Cl^- ions. The (-) isomer is very weakly excitatory at low concentrations and strongly inhibitory at moderate to high concentrations, inhibiting excitability by increasing membrane conductance to Cl^- ions. The Cl^- -dependent inhibitory response to (-) pentobarbital is blocked by the convulsants picrotoxin and bicuculline. Under voltage clamp (-) pentobarbital induces a membrane current response associated with visible fluctuations in the current trace. Spectral analysis of these fluctuations shows that they are well fit by a single Lorentzian equation. The estimated average duration of these drug-induced channel events is about five times that estimated for GABA-activated channels on the same cell. The conductance of these channels is similar to that estimated for GABA. Close examination of the baseline spectra reveals that they are best fit by a $1/f$ relationship with no detectable evidence of an incipient Lorentzian term indicative of the presence of ambient GABA. Thus, despite the fact that (-) pentobarbital activates a Cl^- ion conductance like GABA and is blocked by agents which block GABA, it is not clear how it is "GABA-mimetic". The drug does not appear to be acting by potentiating ambient GABA. One interpretation is that the drug is able to engage GABA receptors and simultaneously modulate the receptor-activated Cl^- conductance. Alternatively, it may engage receptors for an endogenous ligand other than GABA, which are coupled to a Cl^- conductance mechanism or it might be able to activate some Cl^- channel mechanisms directly.

We have continued to examine the pharmacology of benzodiazepines on the excitability of cultured spinal neurons. In addition to the anticonvulsant actions alluded to above we have found three other functionally distinct effects of these drugs. Most of the pharmacology has been carried out using flurazepam. The three other effects include 1) a direct increase in Cl^- ion conductance similar to GABA, 2) an elevation in threshold for single or multiple spike firing and 3) a potentiation of

GABA-mediated Cl^- conductance. Threshold concentration for demonstrating the first effect is 1 pM, making the substance the most potent of those we have tested thus far. Threshold concentration for demonstrating the other two actions and the anticonvulsant effect lies between 100 and 200 pM. The concentration range producing the optimal effect is 500 pM-1nM. Above this range there is a gradual decrease in efficacy for all of the flurazepam actions so that at about 100nM the drug is practically without effect.

9. Defined tissue culture media. A major problem with tissue cultured CNS neurons is the variability of the preparation. One of the important determinants of this variability probably derives from the use of horse serum in the growth media. By using a variety of additives (hormones, minerals, etc.) that have previously been shown to support neuroblastoma cells successfully in culture, we have your tissue cultured neurons in serum-free media for periods up to two months. While the cultures still have to be started in a media with serum, they can be weaned from serum within days. Anatomically and electrophysiologically these cultures mature as well or better than cells grown in serum.

Significance to Biomedical Research and the Program of the Institute: Dissociated cultures of the mammalian central nervous system are proving to be an extremely useful preparation to study the physiology and pharmacology of central neurons. We have begun by focussing on several aspects of receptor pharmacology, examining the membrane effects of amino acids, peptides, purines, pyrimidines, benzodiazepines and barbiturates. We have been able to resolve details of the cellular pharmacology of these effects beyond that which has been shown in vivo. Our research has focused on receptor and membrane pharmacology since this aspect of cell-to-cell communication is relatively easily studied and yet plays a crucial role in intercellular communication and cell excitability. It is clear from these initial studies that a variety of clinically important drugs affect receptor-coupled and membrane changes in excitability and that these changes may well be one of the bases for their pharmacologic effects in the CNS. The results may thus help to provide a more solid scientific basis for the actions of these clinically important drugs.

Proposed course: The project will continue to examine the physiology of intercellular communication in the nervous system and the pharmacology of central receptors and membranes. The neuropharmacology will proceed at the three aforementioned levels of analysis. Structure - activity - relationships of benzodiazepine and barbiturate actions on central neurons, single channel level analysis of receptor function, and correlation of synaptic events with estimates of elementary events are three particular areas of future focus. Such experiments should provide more meaningful and quantitative analyses and a better understanding of the membrane mechanisms underlying the actions of neuroactive substances. Much of the research carried out thus far has utilized unidentified spinal cord and

dorsal root ganglion cells. An important advance that can be made will involve experiments with identified cell types in culture. Three lines of investigation undertaken for this purpose are 1) the enrichment of cultures for specific cell types and 2) the application of immunohistochemical techniques for identifying vital neurons without the use of fixation techniques and 3) the development of defined media for growing tissue cultured cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02330-03 LNP																																				
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SUMMARY OF WORK (200 words or less - underline keywords) Mammalian central neurons have been grown in <u>tissue culture</u> to study 1) <u>immunohistochemically identified peptidergic and GABAergic neurons</u> , 2) <u>opioid peptide synthesis</u> by some of these neurons, and 3) the biochemical characteristics of specific receptors endogenous ligands. Sensory and spinal neurons stain specifically for <u>nerve-specific enolase</u> , while non-neuronal cultured cells do not. Some of the cultured neurons stain positively for either <u>methionine</u> or <u>leucine-enkephalin</u> , <u>dynorphin substance P</u> , <u>somatostatin</u> or <u>glutamic acid decarboxylose (GAD)</u> . Enkephalinergic neurons can synthesize and release methionine-enkephalin when incubated with labelled methionine. Binding studies have demonstrated the existence of several types of receptor on cultured neurons including those for GABA, opioid peptides and benzodiazepines. The results show the utility of using cultured nerve and muscle cells as model systems to study, with neurochemical techniques, the synthesis of endogenous substances and the biochemical characteristics of their receptors.																																						
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Objectives: The objective of this research is to gain insight into the (1) development, dispositions and functionality of central neuronal membrane receptors, (2) mechanisms of peptide synthesis, and (3) physiology of neurons identified immunohistochemically.

Methods Employed: The presence of receptors stereospecific for particular agonists is investigated using conventional receptor binding assay techniques. The mechanisms of peptide synthesis are studied by incubating cultures with radioactive precursor amino acid, extracting radioactively labelled peptides and submitting these to a multi-step purification procedure. Immunohistochemical identification of neurons containing specific antigens is carried out using conventional immunohistochemical fluorescence and "PAP" techniques applied to neurons grown on coverslips in culture.

Major Findings: 1. Immunohistochemistry of cultured neurons. A small fraction of the cells which grow in culture stain positively for nerve-specific enolase, an enzyme marker specific for nerve cells. Most of the cells in culture (fibroblasts and other background elements) do not stain for the enzyme. Intracellular recordings from those cells which stain positively for the enzyme showed that these cells possessed membrane properties characteristic of nerve cells *in vivo*, including excitability and spontaneous synaptic activity. Some elements whose morphology resembles the enolase-positive neurons can be stained, using immunohistochemical methods, for either of five peptides (substance P, somatostatin, dynorphin, leucine or methionine enkephalin) or glutamic acid decarboxylase, the enzyme important in the synthesis of GABA. These elements are presumed to be nerve cells. They do not appear to have distinctive morphologies. We are currently comparing the anatomical disposition of GAD-positive elements on cultured spinal neurons with the functional effects of GABA on the membrane properties of the cell invested with GAD-positive structures. Do GAD-positive structures invest nerve cells in a specific way and how is this investment related to GABA effects on membrane excitability?

2. Peptide synthesis. Spinal cord and brain cultures incubated in radioactive methionine synthesize and secrete methionine-enkephalin. The baseline observations should allow us to ask questions regarding regulation of synthesis and release. One of the next projects will be an examination of precursor-product relationships in cultured neurons. What is the time-course of synthesis? What regulates synthetic rate? Another project will focus on the secretory mechanisms associated with peptide and non-peptide release. What peptides and peptide fragments are released? Are they all functional and if so how do the functions differ?

3. Receptors on cultured cells. Binding assays using labelled ligands including benzodiazepines, GABA and opiates show the presence of stereospecific, saturable binding sites for each type of substance. The functional nature of the binding sites for the drugs has begun to be

approached with electrophysiological measurements of membrane events associated with the pharmacological actions of the drugs on individual nerve cells. What is the developmental biology of the three types of opioid peptide receptor (μ , κ , δ) and how are these receptors related to the three types of chemical excitability?.

Significance to Biomedical Research and the Program of the Institute: Cultured mammalian neurons appear to be a useful system to study receptor pharmacology and peptide synthesis with biochemical techniques. The prime advantages of the preparation are 1) the lack of diffusional barriers to radioactive ligands and precursors and 2) the opportunity to carefully control the extracellular environment. Demonstration of peptide synthesis in vivo has been all but impossible owing to the presence of physical barriers and uptake systems. Likewise binding properties of receptors from in vivo material utilize fractionated membrane suspensions, while the binding experiments in culture can use an intact monolayer of cells. The results achieved thus far contain important baseline observations upon which future questions will be predicated. What regulates the appearance of particular receptors? Are they inserted as completed units or do they mature into functional units while in the membrane? Where are they distributed on nerve cells. How are peptides synthesized and what regulates their synthesis? Finally, the combination of immunohistochemistry with electrophysiology should allow us to examine the physiology of central peptidergic and GABAergic neurons.

Even incomplete answers to the questions raised above will advance our knowledge of the developmental biology of receptors, peptide synthesis and the physiology of identified neurons, since little hard data exists in any of these areas today.

Proposed Course of the Project: The three projects briefly outlined - developmental biology of specific receptors, peptide synthesis and amino acid and peptide immunohistochemistry - will proceed as deliberately as possible. Once baseline observations have been obtained, the first generation of appropriate and important questions will be asked. Some of these questions have been stated in various sections of this report.

Publications

D.E. Schmechel, M.W. Brightman and J.L. Barker, Localization of neuron-specific enolase in mouse spinal neuron grown in tissue culture. Brain Research 181: (1980) 391-400.

J.F. McKelvy, C-J Kin, L. Chang. P. Joseph-Bravo, J-L. Charli, M. Pacheco, M. Paulo, J. Neale and J.L. Barker, Biosynthesis of Brain Peptides in Brain Peptides: A New Endocrinology, A.M. Grotto, E.J. Peck, and A.E. Boyd, eds., Elsevier/North Holland, pp. 182-196 (1979).

M.K. Ticku, A. Huang and J.L. Barker, GABA receptor binding in cultured mammalian spinal cord neurons. Brain Res. 182: (1981) 211-206.

M.K. Ticku, A. Huang, and J.L. Barker, Characterization of γ -aminobutyric acid receptor binding in cultured brain cells. Mol. Pharm. 27 (3): (1981) 285-289.

A. Huang, J.L. Barker, S. Paul, V. Moncada, and P. Skolnick, Characterization of benzodiazepine receptors in primary cultures of fetal mouse brain and spinal cord neurons. Brain Research 190: (1981) 485-492.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02331- 03 LNP
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) A Study of the Complex Receptive Field Properties of Turtle Retinal Neurons		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Henry G. Wagner Chief LNP NINCDS		
COOPERATING UNITS (if any) Prof. P.L. Marchiafava - Lab. Neurophysiology CNR Pisa, Italy		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: .1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) A study of the <u>spectral sensitivities</u> of <u>ganglion cells</u> disclosed two types. About one third of the intracellularly recorded ganglion cells are type A. These show <u>antagonistic color responses</u> . Their dendritic trees are monolayered and connect to <u>bipolar cells</u> . The remaining two thirds of intracellularly recorded cells are type B, do not show color opponency and their dendritic trees diffusely spread throughout the innerplexiform layer. These cells show bipolar and <u>amacrine</u> inputs.		

19 LNP/IRP

Project Description:

Objectives: To characterize the receptive field properties of all cell types in the turtle retina.

Methods Employed: Intracellular and extracellular recordings with glass microelectrodes of the electrical potentials produced by the retinal neurons in response to stationary and moving spots of light are employed. Intracellular injection of dyes is also accomplished with these electrodes to correlate recorded function with structure.

Major Findings: A collaborative study of the spectral sensitivities of amacrine, bipolar and ganglion cells of turtle was made using intracellular microelectrodes. Cells were injected with horseradishperoxidase after recording, processed, mounted and examined microscopically for identification of the neurone. Dendritic trees of these cells were photographed and traced with respect to the innerplexiform layers (IPL) to determine level of synaptic contacts. Two classes of ganglion cells could be distinguished. Type A does show color opponency, spectral max are in the red and in the green; its dendrites are monolayered in the IPL. Type B does not show color opponency with spectral max only in the red. Its dendrites are diffusely layered in the IPL.

Retinal ganglion cells of the turtle retina also show orientation specificity; slits of light when shone upon the retina at the preferred orientation elicit a vigorous response but when shone orthogonal to this orientation evoke little response. Horizontal cells show a similar effect but to a lesser degree. Anatomical studies in the turtle retina using the Golgi technique show that some horizontal cells and some ganglion cells have non-uniform dendritic trees which are up to 2 times longer than they are wide and are oriented parallel to the streak.

Significance to Bio-medical Research and the Program of the Institute: The neurons of the retina have been observed to have quite complex receptive fields. Even though these cells have been studied for over 2 decades the mechanisms by which these receptive fields are generated have yet to be elucidated. Our finding of cell properties in the turtle retina may allow the determination of these mechanisms because all types of retinal neurons can be impaled with intracellular electrodes. The use of intracellular staining will allow the determination of structural-functional correlations in these mechanisms.

Proposed Course of the Project: This project will be terminated with the publication of a report now in preparation. Further work on the turtle will be carried out under Project Z01 NS 02339-04 LNP.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02293-04 LNP
PERIOD COVERED July 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Neural Integration and Processing in the Mammalian Visual System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> PI: H.G. Wagner Chief LNP NINCDS </div>		
CODPERATING UNITS (if any) M.L. Wolbarsht, Duke University J. Ringo, Duke University		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TDAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) A study of the <u>receptive fields</u> of <u>ganglion cells</u> in the <u>cat</u> has been made. Using strong <u>chromatic adaptation</u> , the extent and character of the receptive fields of the several spectral components converging on the ganglion cells has been examined. Central responding areas appear to be well defined. The peripheral areas are not well defined. The spectral component are additive (non-opponent) in the center and in the surround, except for the blue component which does not show opponency rather, it shows one value, either excitation or inhibition in the center and surround.		

Objectives: To study neural interactions and processing in a major sensory system. This study will provide insight on how information is organized and processed by a major neural plexus in preparation for transmittal to a distant neural plexus.

Methods Employed: Anesthetized and curarized intact experimental mammals such as the cat are placed in a special holder for stereotaxic placement of an interocular microelectrode to the retina. A modified maxwellian view optical stimulator permits precise light stimuli to be placed on the retina under direct visualization. Electrical responses are correlated with various parameters of the stimulus.

Major Findings: A study of the Spectral sensitivity of Retinal ganglion cells in the cat has been made using strong chromatic adaptation and spatial localization of the stimulus to the receptive center and/or periphery. The study has shown that three independent and chromatically distinct Cone receptor systems are present and converge on many if not all ganglion cells. Most show simple additivity of color components rather than opponency in the same region of the receptive field. A rare cell did show opponency however. Blue receptor input is found in a high percentage of ganglion cells, but not in opponency between center and periphery.

Significance to Bio-medical Research and the Program of the Institute: This study indicates that the cat has a trichromatic visual system. The presence of three separate wavelength independent systems may be more broadly represented in mammalian species than previously believed.

Proposed Course: This project will be terminated with the publication of a manuscript now in press. Future work, using mammals will be carried out under Project Z01 NS 02339-04 LNP.

Publications:

Crocker, Richard A., James Ringo, Myron L. Wolbarsht and Henry G. Wagner, "Cone Contributions to Cat Retinal Ganglion Cell Receptive Fields. J. Gen. Physiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02152-06 LNP								
PERIOD COVERED October 1, 1979 to September 30 1980										
TITLE OF PROJECT (80 characters or less) Neural Connections in the Retina										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">H. Kolb</td> <td style="width: 35%;">Research Biologist</td> <td style="width: 15%;">LNP NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>H. Wagner</td> <td>Head, Sect. Neuronal Interactions</td> <td>LNP NINCDS</td> </tr> </table>			PI:	H. Kolb	Research Biologist	LNP NINCDS	OTHER:	H. Wagner	Head, Sect. Neuronal Interactions	LNP NINCDS
PI:	H. Kolb	Research Biologist	LNP NINCDS							
OTHER:	H. Wagner	Head, Sect. Neuronal Interactions	LNP NINCDS							
COOPERATING UNITS (if any) R. Nelson, LVE NEI A. Mariani, LVE NEI										
LAB/BRANCH Laboratory of Neurophysiology										
SECTION Section on Neuronal Interactions										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205										
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: .0								
CHECK APPROPRIATE BOX(ES) <table style="width: 100%;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS			
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER								
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS									
SUMMARY OF WORK (200 words or less - underline keywords) To understand the neural circuitry of the vertebrate retina. A manuscript is in preparation.										

Project Description:

Objectives: To understand the neural circuitry of the vertebrate retina.

Methods Employed: 1. Light microscopy of Golgi-impregnated material. 2. Electron microscopy of Golgi-impregnated material. 3. Ultra-thin serial sectioning for electron microscopy. 4. Correlations with intracellular recordings and Procion marking of retinal neurons.

Major Findings: In a collaborative anatomical study of Golgi-impregnated monkey retinas, we have discovered a new type of horizontal cell with a distinctly different appearance from the hitherto described monkey horizontal cell type. The new type II horizontal cell has a profusion of fine, multibranched dendrites ending either in clusters or single terminals, and a short (100-300um length) convoluted axon which occasionally sprouts small clusters of terminals. In contrast, the type I horizontal cell has thick dendritic branches bearing large clusters of terminals and a stout axon which travels a direct course for 2mm before ending in a multibranched axon terminal. Type II horizontal cells have larger dendritic trees than type I cells in the foveal region but smaller dendritic trees than type I horizontal cells in peripheral retina. Golgi-EM of the new type II horizontal cells shows that the dendritic terminals contact cones and possibly some rods, while the groups of terminals on the short axon contact select cones. The photoreceptor connections of the type I monkey horizontal cells are already well documented. Calculations of space constants on the type II horizontal cell axon indicates that it probably behaves as a true axon conducting signals away from the cell body. It is hypothesized that the new HII cell contacts green and blue cones while the old HI cell may contact only red and green cones.

The morphology of physiologically identified neurons of the cat retinas has been determined by comparisons of HRP injected cells with Golgi-impregnated neurons. Cone bipolar cells that respond with a hyperpolarization to light prove to be flat cone bipolars, whereas cone bipolars responding with a depolarization to a flash of light are invaginating cone bipolars. Previously, we have shown that the dendritic branching of ganglion cells either in the upper portion of the IPL where they receive flat cone bipolar input or in the lower portion of the IPL where they receive invaginating cone bipolar input determines whether the ganglion cells will be OFF center or ON center respectively. Thus, the ON/OFF center characteristics of direct cone bipolar/ganglion cell connected pathways in the retina, must originate before the bipolar ganglion cell synapses in the IPL: probably at the cone pedicle to cone bipolar synapses in the OPL.

Several amacrine and ganglion cell types have also been studied with HRP after physiological investigation of their responses to light. Future EM analysis of the synaptic input to such marked cells is planned.

Golgi studies of carp and turtle retinas have been successful and correlations of the different cell types with physiologically identified cells will be possible. The turtle, for example, contains 8-10 bipolar types, 15-18 amacrine cell types and 18-20 ganglion cell types. We know that a large ganglion cell type branching high in the IPL is bipolar dominated whereas a large diffuse ganglion cell type is amacrine dominated from comparisons of these morphological findings with some physiological results in turtle retina. We hope in particular, to find morphological equivalents for orientation selective amacrine and ganglion cells in turtle retina by these methods.

Significance to Bio-medical Research and the Program of the Institute:
Studies of the structure of the retina will provide an understanding of the cells within the retina and will in all probability relate to neural circuitry elsewhere in the CNS. Many programs of this Institute are concerned with the physiology and marking of single retinal neurons and thus knowing the morphology and connectivity of these neurons is essential for our further understanding of visual events.

Proposed Course
The project will be terminated with the publication of a final report.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02339-03 LNP
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Neural Coding and Processing of Information in the Visual System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div> PI: H.G. Wagner K. Hara </div> <div> Acting Chief Special Expert </div> <div> LNP NINCDS LNP NINCDS </div> </div>		
COOPERATING UNITS (if any) H. Blum - DCRT, NIH; H. Spekregjse - Institute of Medical Physics - Netherlands; E.F. MacNichol, Jr. - Marine Biological Laboratory, Woods Hole, MA; M.A. All - University of Montreal		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.5	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS </div> <div> <input type="checkbox"/> (a2) INTERVIEWS </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) An extensive study of the <u>spectral sensitivity</u> of <u>retinal ganglion cells</u> in <u>fish</u> has been made to determine the character and complexity of spectral components driving the ganglion cell. Most ganglion cells of these retinas when isolated in very dim light show a λ max of about 530 nm signifying a rod input. At higher levels of illumination the λ max tends to shift to 650 nm although some cells show a λ max of 500 nm. It is extremely rare to find an initial spectral at 450 nm. The response to illumination may be excitatory ("on" response) or inhibitory ("off" response) usually it is both. Center and surround antagonism is usual. The spectral sensitivity curves may be narrow or broad. Broad curves invariably can be shown to be composed of two or more spectral components each having the same sign (on or off) but different λ max. Narrow curves occasionally show presence of more than one component and the additional component of opposite sign. As many as three spectrally different components can often be found in the same cell. The variety of patterns found suggests color moving in <u>carp</u> retinal ganglion cells is very complex.		

Objectives: To study neural interactions and processing of stimulus information in the visual system. This study will provide insight on how information is organized and processed by a major neural plexus in preparation for transmission to the next neural plexus.

Methods Employed: Isolated, oxygenated retinae of suitable fish such as the carp and goldfish are stimulated by light of known wavelength intensity, duration and spatial configuration. Electrophysiological responses in ganglion cells or other neurones in the visual pathway are detected and analyzed with respect to the various parameters of the stimulus.

Major Findings: Using various sized spots and annuli, precisely controlled spatially, plus intense chromatic adaptation backgrounds, it has been possible sort out a number of spectrally distinct excitatory and inhibitory inputs to the ganglion cells of the carp. The spectral components have sensitivity maxima in the blue (450 nm) green (500 nm) and red (650 nm). Spectrophotometric absorption spectra of single cones were made and showed λ max of (450 nm), (534nm) and (622 nm).

Significance to Bio-medical Research and the Program of the Institute: This study will help understand the processing of neural information in the visual system.

Proposed Course of the Project: A major inquiry into the organization of the receptive fields of ganglion cells, amacrine and bipolar cells of carp goldfish will be made.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02221-05 LNP
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Ionic Mechanisms of Phototransduction in Rods of the Vertebrate Retina		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J. Schnapf Guest Worker NIH National Research Fellow		
COOPERATING UNITS (if any) R.N. McBurney, Department of Physiology, University of Newcastle upon Tyne, England		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.1	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Using a close fitting extracellular pipette electrode over the outer segments of <u>single cones</u> of salamander and <u>turtle retina</u> , an attempt was made to record <u>single photon responses</u> by measuring changes in the membrane current of the outer segments. Successful recordings were made which provide sufficient data to calculate values of 0.012 pA per photon isomerization and a conductance change of 0.67 pS.		
28 LNP/IRP		

Project Description:

Objectives: The principal objective of this study is to characterize the membrane mechanisms that are involved in the transduction of light stimulation into the electrical response of the photoreceptor.

Methods Employed: Suction pipette recordings of electrical potential and conductance were performed in the isolated retina using glass capillary microelectrodes on the outer segments of cones. The retina was mounted in a chamber which allowed superfusion of the photoreceptors with solutions of various ionic compositions. Dissection of the preparation and microelectrode positioning was accomplished using infrared visualization.

Major Findings: The membrane mechanisms by which light is transduced into electrical signals were investigated in photoreceptors. The rod photo-response results from two processes, a light modulated mechanism and a voltage and time dependent mechanism. Techniques for isolating each mechanism and the contribution of each to the photoresponse was studied. The dark potential and the photoresponse were recorded as a function of the concentration of external sodium, potassium and chloride ions as were the effects of 4 amino pyridine, cesium, and ouabain. From these measurements, it is concluded that in the dark, the rod membrane is 10 X more permeable to potassium than to sodium and that at peak of photoresponse, the sodium permeability is reduced by at least a factor of 10. We estimate that the cytoplasmic sodium and potassium concentrations are equal. These ionic gradients are maintained by active Na-K pumps.

Successful recordings were made of changes in the transmembrane current of the outer segments of cones in salamander and turtle. Calculated values of 0.012 pA per photo isomerization and conductance changes of 0.167 pS were obtained.

Significance to Bio-medical Research and the Program of the Institute: This project has significance primarily at the basic research level. Much is known about the ionic mechanisms involved in propagation of electrical signals along the nerve axons and in synaptic transmission. To date an equally lucid description of the primary sensory transducer has not been presented which can account for the known observations. A comparison of the membrane mechanisms involved in the generation of the light response with the membrane properties of axons may provide fundamental insight into the basic mechanisms underlying neuron function.

Proposed Course of the Project: As the principle investigators have departed this project will be terminated after a report is completed and published.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01659-12 LNP
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Synaptic Contacts of Retinal Neurons		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; margin-top: 10px;"> PI: A. Lasansky Research Biologist LNP NINCDS </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Cell Biology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">2</div>	PROFESSIONAL: <div style="text-align: center;">1</div>	OTHER: <div style="text-align: center;">1</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Intracellular injection</u> of chloride ions in <u>retinal cones</u> of the tiger <u>salamander</u> enhances the depolarizing effect of surround illumination. In addition, depolarization by extrinsic currents reverses the polarity of the enhanced surround response. It is concluded that the antagonistic effect of surround illumination is due to a synaptically (chemical) mediated increase in the <u>chloride conductance</u> of the <u>cone cell membrane</u>. </p>		

Objectives: To investigate the fine structure and function of synapses between retinal neurons.

Methods Employed: Electron microscopy combined with silver impregnations by the method of Golgi and intracellular injections of horseradish peroxidase. Electrical recordings with intracellular microelectrodes.

Major Findings: Although retinal photoreceptors are hyperpolarized by light, extending the illuminated area to include their distant periphery may contribute a depolarizing influence, attributed to feed-back from horizontal cells, that reduces the amplitude of the central response. It is not yet known, however, whether this effect is synaptically (chemically) or electrically mediated, nor has it been established whether it is due to changes in the permeability of the photoreceptor cell membrane.

During the present work, the response of salamander cones to steps of light on their surrounding area were intracellularly recorded through micropipettes filled with 2M potassium acetate or 2M potassium chloride. Using 2M potassium acetate, the responses showed a larger relaxation for a 1100 μ m spot than for a 100 μ m spot, but dim annular illumination failed to evoke any detectable response. With 2M potassium chloride, the larger spot or the annulus evoked responses that were mainly or purely depolarizing, respectively, while the responses to the smaller spot were still hyperpolarizing. The depolarizing response to annular illumination had an increased amplitude immediately after discontinuing the injection of inward current through a micropipette filled with 2M potassium chloride. Finally, depolarization by extrinsic current reversed the polarity of the depolarizing response to annular illumination.

It is concluded that the enhancement of the depolarizing influence of the surround is due to an increase in the intracellular concentration of chloride ions, which issue from the electrodes by passive diffusion or electrophoresis. Taking into consideration the effect of depolarizing current, it follows that surround illumination induces an increase in the chloride conductance of the cone membrane.

Significance to Bio-medical Research and the Program of the Institute:

It is hoped that these observations will help in identifying the mechanisms of synaptic transmission between retinal neurons, and provide a better knowledge of the neuronal networks involved in the processing of visual information within the retina.

Proposed Course

An attempt will be made to record intracellularly from cones in the superfused salamander retina. If this can be accomplished, it will be possible to study in more detail the properties of the membrane changes underlying the cone synaptic response. In addition, it should be possible to conduct pharmacological experiments to investigate the identity of the chemical transmitter.

Publications

A. Lasansky. Lateral contacts and interactions of horizontal cell dendrites in the retina of the larval tiger salamander. J. Physiol. 301: 59, 1980.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Biophysics
National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report
October 1, 1979 thru September 30, 1980
National Institute of Neurological and Communicative
Disorders and Stroke
Laboratory of Biophysics
William J. Adelman, Jr., PhD, Chief

INTRODUCTION

The research program of the Laboratory of Biophysics is concerned with investigating molecular and cellular mechanisms responsible for excitation, membrane potentials, the generation of the nerve impulse, synaptic activity, the biophysical basis for the functioning of simple nervous systems, and the cellular basis for such integrative neural functions as behavior and learning. The laboratory makes wide use of physical and chemical techniques, on-line and off-line digital computers and a variety of applied mathematical methods. The laboratory is composed of two units. One of these units operates on a year-round basis at the Marine Biological Laboratory in Woods Hole, Mass. The Woods Hole Unit is composed of 2 sections: the Section on Neural Membranes and the Section on Neural Systems. The Bethesda unit of the laboratory is made up of the Section on Molecular Biophysics.

WOODS HOLE UNIT OF THE LABORATORY OF BIOPHYSICS:

Section on Neural Membranes.

The Section on Neural Membranes uses modern electrophysiological, electron optical, mathematical biophysical, and computer science techniques to investigate the function and structure of neural cells and tissues at limits approaching the molecular level. The general approach is to examine mechanisms that universally and fundamentally underlie all neural function. Emphasis is placed on membrane ionic channel structure and function. Model systems are derived, tested and used to simulate neuronal function under a variety of natural and experimental conditions. Subcellular structures supportive of axoplasmic transport and membrane ionic channel formation are sought. The physical mechanisms involving the structures of muscle and nerve responsible for contraction and mechanotransduction are probed and these are related to both the biochemical and structural elements underlying these mechanisms.

The project on voltage clamp and impedance measurements studied second order admittance in the squid giant axon. This work has been carried on in collaboration with Emory University and the Rockefeller University. Space clamped giant axons were voltage clamped near rest with the wave form $V(t) = V + V_0 \cos(2\pi f_0 t)$. Fourier transformation of the membrane current revealed sinusoidal components at f_0 , $2f_0$, etc. For V_0 near 1 mV and f_0 near the resonant frequency of the membrane, the amplitude of the $2f_0$ component increases with depolarization, decreases with TTX, and varies approximately as V_0^2 . The behavior of the f_0 and $2f_0$ components is in qualitative agreement with the Hodgkin-Huxley equations; however, the null amplitude of the f_0 component, predicted by the HH-equations to occur at particular values of V and f_0 , was not found. Analysis of f_0 and $2f_0$ components for an axon clamped at rest revealed a large peak at 68 Hz corresponding to 113 nA, and a smaller peak at 136 Hz corresponding to 2.9 nA. Decreasing f_0 to 20 Hz in-

creased the $2f_0$ component; increasing f_0 to 200 Hz decreased it. Higher order components were also studied.

In collaboration with the University of Minnesota, this project has also been concerned with membrane excitation properties determined from a statistical evaluation of impulse trains. The space clamped squid axon membrane and two versions of the Hodgkin-Huxley model (the original, and a strongly adapting version) were subjected to a first order dynamic analysis. Stable, repetitive firing was induced by phase-locking nerve impulses to sinusoidal currents. The entrained impulses were then pulse position modulated by additional, small amplitude perturbation sinusoidal currents with respect to which the frequency response of impulse density functions were measured. (Impulse density was defined as the number of impulses per unit time of an ensemble of membranes with each membrane subject to the same stimulus.) Two categories of dynamic response were observed: one showed clear indications of a corner frequency, the other had the corner frequency obscured by dynamics associated with first order conductance perturbations in the interspike interval. The axon membrane responded with first order perturbations whereas the unmodified Hodgkin-Huxley model did not. Quantitative dynamic signatures suggested that the relaxation times of axonal recovery excitation variables are twice as long as those of the corresponding model variables. A number of other quantitative differences between axon and models, including the values of threshold stimuli, were also observed.

The project on the function and structure of ionic channels continues to focus on block by cations. Recently investigated were both sodium and potassium channel block by a much larger quaternary ammonium ion than studied previously. The compound, PTR (phaeantharine chloride), is a bis-benzylisoquinoline quaternary ammonium ion obtained from a tree native to the Philippines. The compound is of interest for two reasons. First, it is considerably larger than previous quaternary ammonium (QA) ions studied since it is composed of multiple, conjugated quinoline groups. Second, it possesses two quaternary nitrogen groups. Earlier work in this laboratory showed that symmetric alkyl QA ions of increasing size block the potassium channel with very nearly the same voltage dependency, indicating that they act at the same apparent location within the membrane field. It is of considerable interest for putting upper limits on possible size of the inner K-channel mouth to see whether PTR blocks and to what extent it penetrates the membrane field in doing so. Analysis of voltage clamp later for steady state current reduction showed that PTR is about an order of magnitude less potent than tetraethylammonium ion (TEA) in blocking the K channel. However, the block appeared to show a similar voltage dependence. More surprising perhaps was that PTR blocks the sodium channel better than the potassium channel.

A phenomenon seen in perfused axons that is not described in the Hodgkin-Huxley model is the persistence of steady state sodium currents at positive membrane potentials. It is of considerable interest to know whether the presence of "foreign" ions induces this behavior. As a preliminary step it is necessary to know whether incomplete inactivation occurs in non-perfused axons. Voltage clamp experiments were performed in which membrane current in intact axons before and after external TTX addition was compared. The difference between these sets of records at 0, +80 and +120 mV revealed a sustained sodium current comparable to that seen in Cs-perfused axons.

A collaborative study of a photoreceptor soma with the Section on Neural Systems was initiated. A two microelectrode voltage clamp technique was used to

explore the behavior of the voltage dependent dark conductances of the type B photoreceptor in H. crassicornis. These photoreceptors possess an early outward current which, from reversal potential measurements, appears to be carried by K ions. The activation of the conductance is voltage dependent and, unlike some gastropod somas, the inactivation which this conductance displays has a voltage dependence to its kinetics, as well as the steady state value.

In another study done under this project, a model of ion translocation across membranes was derived in which ion motion through channels was treated as a random walk on a one-dimensional lattice having some number, s , of ion selective sites ($s \geq 1$). The model was applied to tracer flux measurements, inward (anomalous) rectification, and voltage dependent block of potassium current by cesium and sodium ions in nerve, muscle, and echinoderm egg cell membranes. Inward rectification was modeled by a hypothetical blocking particle located either at a non-blocking site on the inside of the membrane or at one of the sites in the channel. The blocker was assumed to move from site to site exactly like a potassium ion, but it was not allowed to exit the cell into the external medium. Cation blockade of channels was treated in a similar manner. Tracer flux measurements, inward rectification, and Cs block of inward currents were all consistent with a channel having two sites. Reduction of outward current in nerve by internal sodium ions was found to be consistent with block by Na ions at the innermost site which prevents further outward motion. The secondary increase of potassium current at large positive potentials which has been observed under these conditions was modeled by a voltage dependent release of block attributed to passage of Na ions through the channels.

In the project on subcellular and extracellular neural structures, an extensive electron microscopical study was made of the fibrous elements most commonly found in the cytoplasm of both vertebrate and invertebrate neural tissue (f-actin filaments, microtubules, also referred to as neurotubules, and, more characteristically, neurofilaments). These elements are oriented parallel with the elongated parts of neurons such as dendrites and axons. Neurofilaments were shown to possess short lateral projections, which appeared as bridges between neighboring neurofilaments or between neurofilaments and adjacent microtubules.

Improved fixation, particularly of the squid giant axon, was achieved by maintaining conditions favoring the polymerized form of tubulin (i.e. microtubules). In addition to maintaining an appropriate pH level and osmolarity by means of dilute 100 mM phosphate buffer and rather large concentrations of sucrose, care was taken to maintain very low levels of free Ca^{++} by addition of EGTA and to maintain the Mg^{++} concentration at the millimolar level and the temperature of the preparation between 10° and 15°C, conditions which favor the polymerized form of tubulin.

Thicker sections (0.2 - 1 μm) were used and examined using the EM400 electron microscope, the better to observe the detailed three-dimensional distribution of cytoplasmic components such as microtubules, neurofilaments and associated proteins within individual neuronal processes.

Stereo electron microscopic observation of thicker sections demonstrated clearly the presence in both Loligo and Hermissenda axons of a highly ordered neuroplasmic lattice comprising longitudinally oriented ~ 10 nm neurofilaments, thinner 5-6 nm filaments which are possibly actin, and microtubules (when present)

linked together by very thin transverse bridges of unknown nature.

The lattice has extensive properties, with domains of order often extending over several micrometers. In small axons of both Loligo and Hermisenda, the lattice domain often encompasses the whole fiber diameter. The transverse bridges appear to end at, or are structurally continuous with, membranous elements such as the axolemma, vesicles, endoplasmic reticulum and mitochondria. It is thought that some or all of the lattice components are involved in axonal transport processes. These studies have been extended to vertebrate myelinated axons. The crossbridged axoplasmic lattice structure appears more ordered in these vertebrate neurons than in the invertebrates.

In addition, the STEM capability of the EM400 was subjected to line-by-line analysis of electron density profiles of the preparations. Signal averaging, autocorrelation, and Fourier transformation techniques were employed. These methods supported the idea of an ordered neuroplasmic lattice inherent in axoplasm and neuroplasm.

Another aspect of this project was carried out in collaboration with Carnegie-Mellon University. In order to correlate structure with biochemical identification of protein components contributing to these structures, neurofilaments, 10 nm in diameter, from the axoplasm of the squid Loligo pealei, were isolated by a combination of sonication and Millipore filtration. Using this technique, it was shown that actin (43,000 D) and tubulin (56,000 D) are physically separable from intact neurofilaments. The neurofilament-rich retentate contained two major proteins with molecular weights of 200,000 and 63,000.

The project concerned with excitation-contraction processes in skeletal muscle was directed toward resolution of the following specific question. Does the effect observed in resting skeletal muscle which resembles a viscoelastic component continue unchanged during the excitation of the muscle and subsequently remain observable into the fully active contractile state of the muscle? A specific series of experiments were designed to answer this question because such a component might alter the interpretation of instantaneous elasticity measurements previously reported in this laboratory using the method devised and described earlier in this project. The results showed that the effect which resembled a viscoelastic component in resting muscle was actually modified during the activation process and became identified with crossbridge activity in the fully active muscle. Thus, a viscoelastic component of active skeletal muscle had failed to be identified and interpretation of the above result confirmed the suggestion that resting muscle may display an active crossbridge turnover.

The project concerned with mechanoelectrical transduction in nerve was directed toward characterization of the initial membrane response to stretch, which is a depolarization and the subsequent repolarizing and hyperpolarization phases of the response. Specifically, the qualitative relationships between the parameters of the mechanical perturbations and the various phases of the response were sought.

The biphasic character of the membrane potential response to the particular stretch pattern used in this laboratory was well established. The pattern consisted of ramp displacements 3-6 percent of the specimen length completed in about 1 msec, a brief plateau of 1-3 msec duration and an equally rapid return to the initial axon length. Variations in the ramp velocity and amplitude and the

displacement plateau duration have shown the depolarizing phenomena to arise solely during the stretch component of the ramp with the hyperpolarizing phenomena related largely to the plateau duration and amplitude. One of the major differences between the electrical response of squid axon membrane to mechanical strain and that of other isolated axon preparations such as Myxicola and Homarus giant axons can now be accounted for in the different methods used to apply the mechanical perturbations to the membranes. Through the use of tetrodotoxin (TTX), a known sodium channel blocking agent, it was shown that sodium ions are probably involved in the initial depolarization response to stretch. Preliminary interpretations of recent observations have suggested that the repolarizing process which leads to the hyperpolarization component of the biphasic response to stretch is initiated only after sufficient membrane strain and its amplitude and duration are highly dependent on the stretch plateau duration. These findings, when confirmed, may provide a useful means for separating the processes which underlie the initiation of inward and outward currents during membrane activity.

Section on Neural Systems.

The objective of the Section on Neural Systems is to study the mechanisms by which simple neural networks process information with particular emphasis on mechanisms of learning. Information processing involving sensory transduction, synaptic interactions, intersensory communication, conditioning paradigms, membrane and synaptic modification, developmental stages and biochemical mechanisms is of interest. Several marine specimens are used as experimental preparations. The principle preparation is the nudibranch mollusc, Hermisenda crassicornis. Methods of study include electrophysiologic, biochemical, electron microscopic, behavioral and developmental techniques. The following are highlights of the experimental program of the section during this reporting period.

In the area of behavioral and cellular conditioning, several new findings have been made. Complementary behavioral and neurophysiological studies examined stimulus specificity for associative behavioral changes learned by Hermisenda. Paired, but not random presentations of a light step with rotation-produced stimulation of caudal hair cells produced a significant increase in response latency for the animals' movement towards a test light. This associative behavioral change did not occur when cephalic, rather than caudal, hair cells were those stimulated by rotation. Indeed, response latencies were faster for animals receiving paired vs. random presentations of light and cephalic hair cell stimulation and, in some instances, faster than original baseline latencies.

Intracellular recordings from Type B photoreceptors in the isolated nervous system indicated the enhancement of a long-lasting membrane depolarization (LLD) under stimulus presentation regimens paralleling those involved in training of intact animals. A sensitivity of cumulative photoreceptor depolarization to the quality of hair cell stimulation was shown to derive from the differences in synaptic effects of hair cells upon Type B photoreceptors for caudal vs. cephalic hair cell stimulation. The magnitude and duration of Type B depolarization following light paired with rotation-produced stimulation of caudal hair cells is enhanced by two sources: increased synaptic excitatory input from the ipsilateral optic ganglion and facilitated disinhibition (from caudal hair cells) of the Type B photoreceptor.

Other studies demonstrated that long-term (> 2 days) neural changes in the Type B cell were closely correlated with long-term associative learning of Hermisenda.

Neural correlate studies have been extended to the motor systems of the visual pathways. Long-term changes of motor neurons (output) specific to associative learning behavior can now be entirely accounted for by differences of Type B input to these cells.

In the area of neural network analysis, a study of motor systems within sensory pathways has made use of intracellular recording and marking techniques to identify large pedal motor neurons within the visual pathway. Intracellular electrophysiological recordings and fluorescent dye iontophoresis indicate that there are ocular and extraocular inputs to putative motor units in Hermisenda and suggest that the cellular response to light may be lateralized.

A new technique for complete reconstruction of individual neurons with conventional microscopic visualization was developed. A specified neuron's voltage responses were first electrophysiologically recorded, and horseradish peroxidase (HRP) was then iontophoresed. By passing the fixed tissue through a series of histological clearing treatments the surrounding tissue became almost transparent while the injected neuron was filled with a light-absorbing reaction product. Using high resolution Differential Interference Contrast microscopy, the entire specimen was optically sectioned. In each optical section the filled neuron was visually distinct from the surrounding tissue.

Qualitative and quantitative calcium ion studies on the pigment cells and Type B photoreceptors of the adult eye using electron-probe analyses were undertaken to detect changes in intracellular calcium levels and binding sites in adult animals experiencing different training paradigms.

In the area of receptor physiology, power spectra of the current noise in hair cells (obtained in voltage-clamped hair cells) have provided more accurate values of hair cell conductances at rest and during stimulation. These data supported the application of a shot noise model to mechanotransduction by Hermisenda hair cells. The power density spectrum of voltage noise observed in these cells during steady state stimulation could be fit with the product of two Lorentzians where τ_1 was similar to the inverse of the rate constant determined from relaxation measurements and τ_2 was the membrane time constant.

In other hair cell experiments, vanadate (+5) anion, a reversible inhibitor of dynein crossbridge cycling injected into hair cells, initially caused the cilia to lose their normally upright, rigid, vibratile form and assume a more classic, pliable beat pattern. Voltage noise decreased as the cilia slowed and bent more extremely, nearly disappearing as motility was lost. When the intracellular vanadate concentration approached 10^{-5} M, the cilia were arrested in an effective stroke against the cell membrane. The cell no longer depolarized upon gravitational or local mechanical stimulation. Rapid reversal of ciliary inhibition with norepinephrine or slow reversal with time restored both the voltage noise and depolarization response. Cilia were rendered rigid and upright by covalent crosslinkage of their membrane "sleeve" to the 9 + 2 axoneme, using the photoactivated, lipophilic, bifunctional agent 4,4'-dithiobisphenyl azide. In the initial stages of crosslinkage, the cilia remained vibratile but slowed and moved through wider excursions. Voltage noise decreased in frequency but increased in amplitude. When the cilia were fully arrested, voltage noise was minimized while the resting potential and membrane resistance remained essentially constant. Mechanical stimulation of the rigid cilia, normal to the cell membrane, elicited a generator potential of the same amplitude but of greater duration than

before treatment. Since cilia which are partially arrested by vanadate undergo increased binding but the hair cell shows decreased noise, neither the axoneme nor the ciliary membrane proper would appear to be sites of direct transduction. In full vanadate arrest, the exposed plasma membrane itself shows no response to stimulation. In cells with beating, stiffened cilia, however, the voltage noise becomes amplified, implying an increased efficiency of transduction. These data indicate that active but rigid flexure of the axoneme near the base of the cilium is required for continuous transduction. The ciliary necklace region is the most likely transduction site, being the terminal leverage point through which force is applied to the membrane via the flexing ciliary shaft.

In collaboration with the Section on Neural Membranes, a voltage clamp study of the Type B photoreceptor was carried out to characterize light and voltage dependent conductances. Step clamp commands from a holding potential of -60 mV elicited an early, transient outward current that appeared to be carried predominantly by K^+ ions. Both steady state activation (peak value) and inactivation of the conductance were voltage dependent. Activation showed a maximum, "e"-fold change of 12.5 mV over the range of -20 to +10 mV. The outward current showed inactivation from a relative value of 1 at -70 mV to zero at about -10 mV. -30 mV is the voltage at which the current is half inactivated at steady state. The kinetics of inactivation, like the steady state value, showed voltage dependence. The decline of the outward current during sustained depolarization was fitted by two exponentials, one with a comparatively short time constant which declines from about 130 msec at -20 mV to about 50 msec at +10 mV. The early outward current was strongly activated at the time that the light induced sodium current was rising. The temporal position of these two currents suggested that possible extrinsic control of resting potential by paired stimulus presentations could influence the rising phase of the generator potential by setting the degree of early outward current inactivation.

In the neurochemistry studies, protein phosphorylation in the eyes of animals from trained, unpaired, random, and normal control groups were examined. Seven phosphoprotein bands were detected in the eyes from all animals. In paired (trained) vs. control groups, there were no significant differences in five of the phosphoprotein bands. However, significant overall differences between paired (trained) and control groups ($p < 0.01$) were found for bands with approximate MW of 23,000 and 20,000 daltons. Post-hoc comparisons revealed that the paired group was significantly different from both the random and unpaired control groups ($p < 0.01$) while the controls were not different from each other. One phosphoprotein band (approx. MW = 23,000) was increased 49% in paired as compared to random controls. The second phosphoprotein band (approx. MW = 20,000) was increased 56% in paired as compared to random controls. The changes in the two phosphoprotein bands, as mediated perhaps by protein kinases and/or phosphatases, may be related to the increase in input resistance in Type B photoreceptors observed in trained animals and to the increase in the long-lasting depolarization in Type B photoreceptors observed in isolated nervous systems following pairing.

In the study of neural development, serial electron microscopic sections of developing forms permitted accurate staging of sensory pathway formation in Hermisenda. Differentiation of neural tissue into the hair cells of the statocyst has been followed from approximately eight days post-fertilization. Formation of photoreceptors, optic ganglion and chemosensory organs was also followed with serial sections. Detailed comparisons of cellular and subcellular speciali-

zations of neural elements within these pathways of trained and control animals have also been initiated.

BETHESDA UNIT OF LB:

Section on Molecular Biophysics.

The Section on Molecular Biophysics is involved in four general areas of research: molecular mechanisms of channel gating, properties of open channels, molecular mechanisms of drug action, and biological applications of membrane biophysics. The following are highlights of the results of this research program.

In the project on pharmacology and ionic selectivity of membrane ionic channels, the external action of tetraethylammonium ion (TEA) on the membrane of invertebrate axons has been investigated. Using voltage clamp, externally applied TEA was found to have minimal effects on transient sodium currents and to suppress steady-state potassium currents in Myxicola giant axons by causing a specific decrease in the maximum potassium conductance g_K . The dose-response curve suggested an one-to-one stoichiometry for TEA-receptor binding with an apparent dissociation constant of 24 mM. The suppression of I_K was essentially reversible. Experiments performed on high external potassium ion concentrations indicated that both outward and inward I_K were blocked by external TEA. These results suggested the presence of TEA receptors on the outer surface of Myxicola axonal membrane similar to that reported in the frog node.

When resting sodium and potassium conductances were blocked by tetrodotoxin and tetraethylammonium ions respectively, the apparent reversal potential for leakage current in the Myxicola giant axon was found to be depolarized approximately 25 mV with respect to the normal resting potential and to shift towards zero when the membrane potential was depolarized in the presence of external potassium ions. The average Q_{10} for leakage current was 1.63 ± 0.16 .

Work has continued in collaboration with the University of California at Los Angeles on the relation between gating currents and voltage dependent sodium conductance changes in the squid giant axon. Data on slow inactivation were analyzed and these were correlated with a new "16 state" model. The system for the collection of live squid at UCLA has been improved but very rainy weather in January, 1980, interfered with work there. As a result, a trip to the MBL was organized for April 25 - July 1, 1980, which was very productive, particularly in the areas of correlation between gating currents and sodium conductance, slow inactivation, effects of Zn^{++} and comparison between time and frequency domain studies of gating currents.

The project on mathematical modeling has collaborated with LN/NIA so as to describe a compartmental model for the entry of drugs into the brain and cerebral spinal fluid, with good agreement with the experiment. Work has continued on generalizing a model for an ion in a cylindrical pore in a dielectric membrane. New programs have been written to plot contours of electric potential and to introduce the effect of a charge on the wall of the pore. Equations have been derived for the second and third order nonlinear membrane currents of the Hodgkin-Huxley equations under sinusoidal voltage clamp. Computations show qualitative agreements with results of experiments by the Section on Neural Membranes.

The project on voltage-dependent ionic conductance in membranes has studied a model of drug channel interaction in the squid axon membrane. Yohimbine exhibits both a use-dependent as well as a tonic on non use-dependent inhibition of the sodium currents. At present, the structure-activity relationship of various yohimbine analogs, specifically synthesized for this purpose, is being studied. The cis-trans isomers of the various groups have been tested and it was found that β yohimbine and yohimbyl alcohol exhibit the same approximate quantitative effects as yohimbine does. Two other drugs as well also exhibit use-dependent inhibition. They are phenytoin, an anti-arrhythmic drug, and perhexiline, an anti-anginal drug. The dose-response curve of phenytoin was shifted to the right when the membrane voltage was held at a higher potential, i.e., the sodium currents increased for the same phenytoin concentration when the membrane was hyperpolarized. In contrast to yohimbine use-dependence, the use-dependent inhibition by phenytoin increased significantly when the depolarizing pulse duration was increased. The action of perhexiline has been theorized to be due to its calcium antagonism. Calcium shifts the relation of h_{∞} to potential along the potential axis. Perhexiline produces no such shift. Therefore, the action of perhexiline is probably not due to calcium antagonism. Perhexiline seems to work close to the internal membrane, since its action is rapid when internally perfused and is extremely slow when externally applied.

The project on structure and function of the perineurium has progressed to the point where a paper on sucrose permeability of the perineurium has appeared in print and another on the permeabilities of Na^+ , K^+ and Cl^- , measured simultaneously, is in press. The results of impedance measurements and permeability changes during Wallerian degeneration has reached a satisfactory conclusion.

The project on comparison of different modes of axonal stimulation has examined mechanical transduction, a process whereby a mechanical stimulus applied to a cell (neuronal) membrane is converted into a local electrical potential. Myxicola giant axons under voltage clamp were mechanically stimulated. The resultant changes in membrane current depended on both the membrane potential and the amplitude of the mechanical stimulus. The reversal potential of the membrane current-voltage relation was between about -36 mV and +7 mV, becoming more depolarized as the stimulus amplitude increased. The change in reversal potential as a function of mechanical stimulus amplitude corresponds to a change in selectivity. This finding was found consistent with the hypothesis that the mechanically stimulated ionic pathways gradually increase in diameter as the stimulus amplitude increases, but several other possible explanations were offered. One explanation put forth suggested that the pathways activated by mechanical stimuli are specific protein channels, that these channels have several different open conformations, and that increasing the magnitude of a mechanical stimulus preferentially excites less selective conformations.

Another project studied voltage oscillations in the barnacle giant muscle fiber. Barnacle muscle fibers were subjected to constant current stimulation to produce a variety of types of oscillatory behavior when the internal medium contained the Ca^{++} chelator EGTA. Oscillations were abolished when Ca^{++} was removed from the external medium or when the K^+ conductance was blocked. Available voltage clamp data indicated that the cell active conductance systems are exceptionally simple. Given the great complexity of barnacle fiber voltage behavior, this seemed paradoxical. An analysis was carried out of the possible modes of behavior available to a system of two non-inactivating conductance mechanisms. This analysis indicated a good correspondence to the types of

behavior exhibited by the barnacle fiber. The differential equations of a simple equivalent circuit for the fiber were dealt with using some of the mathematical techniques of nonlinear mechanics. General features of the system were a propensity to produce damped or sustained oscillations over a rather broad parameter range, and considerable latitudes in the shape of the oscillatory potential. It was concluded that for cells subject to changeable parameters (either from cell to cell or with time during cellular activity) a system dominated by two non-inactivating conductances could be the source of a rich repertoire of voltage behavior.

The project on excitable membranes of tissue-cultured nerve and muscle cells has examined single post-synaptic channel currents in tissue-cultured muscle. Some of the most compelling evidence for the existence of ionic channels in cell membranes comes from direct recording of quantified current jumps generated by the opening and closing of individual channels. Single channel jumps have been extensively studied for lipid bilayer membranes doped with various channel-forming additives. Recently, agonist-induced single channel currents were detected in denervated frog muscle by use of extracellular electrodes which can isolate the current from a small area of membrane. The current jumps provide a means for the direct test of many of the inferences about ionic channels which have come from electrical noise analyses. Measurements of single channel currents induced by the agonist carbamylcholine in tissue-cultured mammalian muscle were made. These measurements confirmed the earlier noise studies on tissue culture preparations.

In another study, an extracellular patch electrode was used to record ionic currents through individual complement channels in the membranes of antibody coated skeletal muscle. The amplitude of the single channel currents lead to an estimate of 90 pS for the unit conductance. An electrolyte-filled pore having this conductance would have a diameter of approximately 8-10Å. A cation conductance of this magnitude was shown to be consistent with the one-hit theory of complement lysis. The kinetics of channel opening and closing showed marked variability and complexity. It was shown that a channel can flicker open and closed repeatedly indicating that once the attack complex forms, it undergoes rapid structural transitions between discrete open and closed states.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01950-09 LB
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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Excitable Membrane Characteristics: Voltage Clamp and Impedance Measurements.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.8	OTHER: 0.2
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The general aim of this project has been to study excitable membrane characteristics by a variety of physical methods. One aspect has been to improve electrical measurements of excitable membrane characteristics consistent with physical and chemical methods for the study of nerve membrane ionic channels. Two major approaches are used. The first involves the development of methods for analysis of ionic channel admittances and/or conductances by means of oscillating potential voltage clamp techniques. Programs for carrying out this analysis are developed. The second approach involves analysis of excitable membrane characteristics by means of frequency analysis of sinusoidally modulated action potential trains locked to a larger amplitude, higher frequency sinusoidal stimulus. Improvement of bridge impedance techniques to achieve greater accuracy are made. Admittance measurements on giant axons and an investigation of effects of polarizations for comparison with ion conduction models are carried out. This project is supportive of a number of other projects in terms of the development of relevant hardware and software.

Project Description:Objectives.

1) To develop and test a simple method for continuously monitoring membrane instantaneous conductance during a voltage clamp, and to apply this method to determine anomalous potassium channel gating rates as functions of calcium and potassium ion concentrations.

2) Excitation properties of axon membrane from a statistical evaluation of impulse trains. Transfer functions resulting from small amplitude sinusoidal perturbations of stimulus currents responsible for steady state impulse trains are compared with similar functions of excitation models and evaluated in terms of membrane excitation parameters.

3) To determine the admittance of excitable membranes during voltage clamping.

Methods employed.

1) G_K kinetics were obtained by superposing a small jump (ric-rac) voltage, ΔE , on the larger voltage pulse. In this case, $g_K = \Delta I / \Delta E$, ΔI being the corresponding small step in current. Cleaned, single giant axons from the hindmost stellar nerve of the squid, Loligo pealei, were used in this study. Internally perfused axons were voltage clamped using the "ric-rac" voltage pulse technique. Internal solutions were Na^+ -free and contained KF; K Glutamate; K_2HPO_4 in concentrations of 50 mM; 200 mM; 25 mM respectively for a total K^+ -concentration of 300 mM plus 505 mM sucrose to maintain osmotic strength. To obtain total K^+ -concentrations of 150 and 450 mM, the same proportions of potassium compounds were used, plus sucrose concentrations of 792.5 and 217.5 mM respectively. pH was adjusted to 7.2 with free glutamic acid.

External solutions contained NaCl, $CaCl_2$, KCl, 20 mM Tris buffer and $0.1 \mu M$ TTX to block the Na-channels completely. Experiments were performed with 12 different combinations of external Ca^{++} - and K^+ -concentrations ($[Ca^{++}] = 2, 10, 40, 100$ mM and $[K^+] = 5, 10, 50$ mM) with Na-ions used as a trade-off for calcium and potassium to maintain ionic strength.

The main voltage clamp pulses consisted of depolarizations from a holding potential of -60 mV to -10, +5, +30, and +55 mV axointernal relative to external ground with pulse durations of 18 milliseconds, and a resting period of at least 8 seconds between pulses. Ric-rac consisted of square wave voltage clamp steps, centered on the main clamp voltage with 5 mV peak-to-peak amplitude and a square wave frequency of 2 kHz. A frequency, f , of 2 kHz was chosen so as to be less than expected values of $2/\tau_n$, and to be such that the membrane capacitive current transient responses decayed significantly toward zero in times much less than $1/2f$. The ric-rac amplitude was so chosen to assure that the \pm current traces were equidistant from a central current trace generated in the absence of ric-rac. Potassium conductance, g_K , was calculated from the measured $\Delta I(t)$ as $g_K(t) \equiv \Delta I(t) / (5 \text{ mV})$ corrected for a standard 2 ohm-cm^2 series resistance. The experimental temperature was maintained at $4.6 \pm 0.3^\circ C$.

2) Intact cleaned, single giant squid axons were externally bathed in artificial seawater. The axons were current-clamped using a sinusoidal current stimulation rather than short duration square wave currents. A locking sinusoidal current of the form given by Eq. 1 with $a = 1.0$ was adjusted to assure the production of one spike per cycle at $f_0 = 100$ Hz, and typically this required $I_0 = 80$ $\mu\text{A}/\text{cm}^2$. A second, small amplitude-modulating sinusoidal current (of amplitude $I_0/4$) was linearly summed with the locking current to produce a sinusoidal variation of impulse density over a modulating cycle. Action potentials produced by the axon and sync pulses marking each cycle of the modulating sinusoid were treated as all-or-none events that were timed on-line by a PDP-11/10 laboratory computer. Data consisted of clock times (to the nearest 0.2 ms) corresponding to each action potential and sync pulse for a total of at least 200 events for each modulating frequency tested. The data were stored on binary magnetic tape for later processing on an IBM 1800 computer. This consisted of estimating impulse density sinusoids and computing gain and phase for Bode plots. For this study, the input modulation was held constant (25%) and the "gains" reported therefore represent only changes in output modulation. They were computed as $20 \log$ (modulation amplitude). Model impulse trains were generated by numerical integration using a library Runge-Kutta routine on a CDC Cyber 74 computer and a continuous system modeling program on the IBM 1800 computer.

A statistical analysis of nerve-impulse trains produced by sinusoidally modulated current stimuli has been used in connection with certain well-defined models to describe the behavior of membrane properties in the interspike interval. A train of action potentials can be induced by a sinusoidal current having a frequency equal to the impulse repetition rate, f_0 .

$$I(t) = I_0\{1 + a \cdot \sin(2\pi f_0 t)\} \quad (\text{Eq. 1})$$

The amplitude of the sinusoid (equal to aI_0 in Eq. 1) was sufficiently large so that the impulse repetition rate followed the sinusoidal frequency when the latter was changed within a neighborhood of f_0 — that is, the impulses were phase-locked to the stimulus sinusoid. Calculations are made from measured impulse occurrence times when the steady-state repetitive discharge is modulated by adding to the stimulus current a sinusoidal perturbation with frequency $f_m < f_0$ and of small magnitude so that the modulation is of the order of 10-25%. When the unperturbed stimulus is as in Eq. 1, the total stimulus current density then has the form

$$I(t) = I_0\{1 + a \cdot \sin(2\pi f_0 t)\} + I_1 \sin(2\pi f_m t + \quad). \quad (\text{Eq. 2})$$

Estimates of impulse density over a single perturbation cycle are made at a number of different perturbation frequencies. The amplitude, A , of the modulation is defined as one-half the total change in impulse density over a single cycle. This value is converted into a "gain" in decibels, where

$$\text{Gain} \stackrel{\text{def}}{=} 20 \log_{10} kA \quad (\text{Eq. 3})$$

The constant k has a value of 1 with units of (amplitude) $^{-1}$. The phase of the impulse density is determined with respect to the modulating sinusoid and is given in degrees of lead (+) or lag (-). Gain and phase are plotted as functions of \log modulation frequency, f_m , to form Bode plots. Those plots showed certain

features that relate specifically to the membrane parameter

$$\gamma \stackrel{\text{def}}{=} g_m / C_m \quad (\text{Eq. 4})$$

for the interspike interval.

3) Space clamped giant axons were voltage clamped near rest with the wave form $V(t) = V + V_0 \cos(2\pi f_0 t)$. Fourier transformations of the membrane currents were performed. Power spectra were plotted and analyzed and current frequency peak determined.

Major Findings.

1) The time to half-maximum, $t_{1/2}$, of K-conductances, g_K , under voltage clamp were shown to be functions of external Ca^{++} -concentration. "Half-maximum" of g_K was defined as the midpoint between the conductance immediately following depolarization and the conductance at 18 ms. $t_{1/2}$ values were measured for four conditions of bulk K-ionic strengths or transmembrane K-ionic gradients. For a given ionic environment and test potential, the values of g_K at $t = 18$ ms varied somewhat among axons, but $t_{1/2}$ values remained remarkably constant. For equally spaced test potentials, -20, +5, +30, and +55 mV, intraaxonal relative to external ground, an increase in $t_{1/2}$ with increasing $[\text{Ca}^{++}]_e$ for both $[\text{K}^+]_i/[\text{K}^+]_e = 300 \text{ mM}/10 \text{ mM}$ and $450 \text{ mM}/5 \text{ mM}$ was found. The positive slope of $t_{1/2}$ values vs. $[\text{Ca}^{++}]_e$ was abolished and became negative for $[\text{K}^+]_i/[\text{K}^+]_e = 300 \text{ mM}/50 \text{ mM}$ and $[\text{K}^+]_i/[\text{K}^+]_e = 150 \text{ mM}/50 \text{ mM}$. The shifts in $t_{1/2}$ values were within the range of 10 to 15 millivolts for a 5-fold change in $[\text{Ca}^{++}]_e$ for both $[\text{K}^+]_i/[\text{K}^+]_e = 300/10$ and $[\text{K}^+]_i/[\text{K}^+]_e = 150/50$ with the shifts going in opposite directions for these two ionic gradients. Furthermore, with the exception of small increases in the values of $t_{1/2}$ for $[\text{K}^+]_i/[\text{K}^+]_e = 300 \text{ mM}/50 \text{ mM}$, all $t_{1/2}$ values for a given test potential were equal for a calcium concentration of approximately 25 mM.

The degree of sigmoidality of the K-conductance as a function of time appeared to be a continuous function of both $[\text{Ca}^{++}]_e$ and $[\text{K}^+]_i/[\text{K}^+]_e$. For each set the initial delay (or initial slope) before the upturn to maximum rate-of-rise was found to be a systematic (monotonic) function of the Ca^{++} -concentration. The trend of this function (monotonically increasing or decreasing) was in opposite directions for $[\text{K}^+]_i/[\text{K}^+]_e = 450 \text{ mM}/5 \text{ mM}$ and for $150 \text{ mM}/50 \text{ mM}$; the initial slope was greater for the smaller $t_{1/2}$ in each case. The data therefore showed a direct correlation between the amount of sigmoidal inflexion and the slope of the $t_{1/2}$ curves as functions of $[\text{Ca}^{++}]_e$.

A direct comparison between the present data and the Hodgkin-Huxley model K-conductance modified by Adelman and FitzHugh to include the effects of periaxonal accumulation on the determination of \bar{g}_K , α_n , and β_n by Adelman, Palti and Senft showed that the rise-time and sigmoidality were comparable with the experimental data interpolated to $[\text{Ca}^{++}]_e = 25 \text{ mM}$ and the model operated at 4.6°C (the experimental temperature). Model curves were fitted under voltage clamp with $\bar{g}_K = 60.0 \text{ mmho/cm}^2$,

$$\alpha_n(E) = \frac{0.004651(E + 52.07)}{1 - \exp\{-(E + 52.07)/7.93\}}$$

and

$$\beta_n(E) = (0.1011)\exp\{-(E + 60)/26.72\}$$

and interpolated data points for $[Ca^{++}]_e = 25$ mM and $[K^+]_i/[K^+]_e = 300$ mM/10 mM. Making use of this data and invoking calcium surface charge screening, an explanation was developed using a reciprocal interaction hypothesis between a conducting channel ion and channel gate-charges.

2) At the operating temperature of 6.3°C most healthy axons could be made to phase-lock at the rate of $f_0 = 100$ impulses/s in response to sinusoidal current density as in Eq. 1 with $a = 1.0$. The minimum value of I_0 necessary for this response was ~ 75 $\mu A/cm^2$. All axons capable of this repetitive rate were also able to maintain one-to-one phase locking for $f_0 = 75, 50, 25$, and 10 Hz. The minimum required magnitude of I_0 declined steeply with f_0 to a plateau of ~ 30 $\mu A/cm^2$ for f_0 below 50 Hz. A majority of the axons continued repetitive firing over the entire range of modulation frequencies (no dropped spikes) when the locking signal (Eq. 1) is modulated by 25%.

For very low modulation frequencies, f_m , all phase curves tended to a phase advance of 90° (relative to the perturbation sine wave) with the exception of an occasional axon firing at $f_0 = 100$ Hz which tended towards a 270° advance. Depending on the firing rate, and in particular for higher f_0 , the phase advance increased in the midrange of modulation frequencies, f_m , to reach a maximum somewhere in the area of $10 \lesssim f_m \lesssim 25$ Hz. The phase advance again decreased to well below 90° as f_m approached f_0 . The midrange phase advance was invariably accompanied by a direct increase in the slope of the gain curve, which is a constant 6 dB/octave for the lower modulation frequencies.

Further dynamics data of the squid axon were obtained for a wide range of temperatures and found to be strongly temperature-dependent. The overall dynamic characteristics appeared to simply shift to higher frequencies, f_0 and f_m , for higher temperatures.

Axons were able to maintain a phase-locked impulse rate of 150 impulses/s at 12°C and 200 impulses/s at 18°C. These dynamic response properties are consistent with the strong temperature dependence ($Q_{10} = 2.7-3.0$) of the rate constants of the excitable conductance channels and indicate that the dynamics are a direct function of the channel kinetics. Two categories of dynamic response were observed: one showed clear indications of a corner frequency, the other had a corner frequency obscured by dynamics associated with first order conductance perturbations in the interspike interval. The axon membrane responded with first order perturbations whereas the unmodified Hodgkin-Huxley model did not. Quantitative dynamic signatures suggested that the relaxation times of axonal recovery excitation variables are twice as long as those of the corresponding model variables. A number of other quantitative differences between axon and models, including the values of threshold stimuli, were also observed.

3) Fourier transformations of voltage clamped membrane currents in response to sinusoidal membrane voltages revealed sinusoidal components at f_0 , $2f_0$, etc. For V_0 near 1 mV and f_0 near the resonant frequency of the membrane, the amplitude of the $2f_0$ component increased with depolarization, decreased with TTX and varied approximately as V_0^2 . The behavior of the f_0 and $2f_0$ components was in qualitative agreement with the Hodgkin-Huxley equations; however, the null amplitude of the f_0 component, predicted by the HH-equations to occur at particular values of V and f_0 , was not found. Both f_0 and $2f_0$ components for an axon clamped at rest were found. The larger peak at 68 Hz (f_0) corresponded to 113 nA, the smaller ($2f_0$) peak at 136 Hz corresponded to 2.9 nA. Decreasing f_0 to 20 Hz increased the $2f_0$ component; increasing f_0 to 200 Hz decreased it. Higher order components were also studied.

Publications:

Fohlmeister, J.F., Adelman, W.J., Jr., and Poppele, R.E.: Excitation Properties of the Squid Axon Membrane and Model Systems with Current Stimulation: Statistical Evaluation and Comparison. Biophys. J. 30: 79-98, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02087-07 LB																
PERIOD COVERED October 1, 1979 to September 30, 1980																		
TITLE OF PROJECT (80 characters or less) Function and Structure of Ionic Channels: Ion Interactions and Gating Mechanisms.																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
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SUMMARY OF WORK (200 words or less - underline keywords) <u>Voltage clamp</u> experiments are employed to determine functional and structural characteristics of <u>ionic channels</u> in the squid giant axon. Information concerning these characteristics of the ionic channels is gained by studying the interaction of ions which <u>block</u> the passage of normal charge carriers and by studying the effect of <u>voltage</u> upon the opening and closing (" <u>gating</u> ") of channels.																		

Project Description:Objectives.

- 1) To characterize block of both potassium and sodium channels by a large organic ion bearing two identical cationic sites.
- 2) To determine whether the phenomenon of incomplete inactivation of the sodium channel at positive potentials occurs in axons containing native axoplasm.

Methods Employed.

The excitable properties of squid giant axon are studied with the aid of voltage clamp methodology. When desirable, the internal composition of the axon may be manipulated through the use of an internal perfusion system. Extensive use is made of digital techniques for control of the experiment and data analysis.

Major Findings.

1) Phaeantharine chloride (PTR) is a bis-benzyl isoquinoline quaternary ammonium ion. When present in millimolar concentrations inside the axon it blocks both sodium and potassium channels. The characteristics of the block are similar to those of other single quaternary ammonium ions studied in this laboratory. However, it is a more potent sodium channel blocker by almost an order of magnitude. This behavior contrasts with the behavior of the smaller QA ions studied (a series of symmetric QA ions from tetramethylammonium to tetrapentylammonium). In addition, although the ion bears two identical QA groups at either end of a planar ring structure, it blocks the K channel with about the same voltage dependence as tetraethylammonium ion, a far smaller ion. This result may suggest that only one end of the ion may enter the transmembrane field or that the charge moved in the field is independent of the blocking ion.

2) When squid axons are perfused with solutions containing Cs or TMA ions, or when K ions are absent, the sodium conductance is seen not to inactivate fully at potentials more positive than zero millivolts. This observation is at variance with the predictions of the successful Hodgkin-Huxley model. The model is based on observations upon non-perfused axons. These more recent observations suggest that the partial inactivation is caused by the presence of "foreign" ions. The hypothesis was tested by comparing current records for non-perfused axons pulsed to positive potentials. One set of current records was obtained after tetrodotoxin (TTX) was added to the bath to block all sodium channel current. Sodium current measurements obtained this way agree with current measurements with Cs present internally. We conclude that the sodium conductance does not inactivate fully in non-perfused axons.

Publications:

French, R.J. and Shoukimas, J.J.: Blockage of squid axon potassium conductance by internal tetra-n-alkylammonium ions of various sizes. Biophys. J. (In press).

Shoukimas, J.J. and French, R.J.: Incomplete inactivation of sodium currents in non-perfused squid axon. Biophys. J. 32: 857-862, 1980.

Project Description:Objectives.

- 1) To examine the structure and possible function of the major filamentous components found in the cytoplasm of neuronal cells: microtubules, neurofilaments, actin filaments and transverse bridges, and to study their ordered association into a transverse bridge lattice system; to investigate the role of this lattice and its possible functional involvement in axoplasmic transport phenomena.
- 2) To study the protein composition of axonal and neuronal cytoplasm, and to identify specific proteins with each of the filamentous structures referred to above.

Methods employed.

1) This study utilized thicker sections (0.1-0.3 μm) for stereoscopic study of neural networks in conventional TEM (Philips 300) where the upper limit of section thickness is determined primarily by loss of resolution resulting from inadequate imaging of inelastically scattered electrons and by problems associated with heavy metal staining. Improved fixation was observed for squid neural tissue (including giant axons) and for the CNS of Hermisenda crassicornis when the initial glutaraldehyde (2-4%) phosphate-buffered fixative (pH 7.2) contained EGTA (2-40 mM) and Mg^{++} (1-5 mM) with the total osmolarity adjusted with sucrose to not less than 1100 mosm. Initial fixation (30 min.-2 hours) was followed by cold OsO_4 treatment (30 min.-2 hrs.). Specimens were dehydrated and embedded using Epon 812. Specimens were stained en bloc with either aqueous or alcoholic solutions of uranyl acetate, sometimes followed by alcoholic PTA. Often these thicker sections required further staining, usually alcoholic uranyl acetate and aqueous lead citrate for adequate contrast. These methods have also been successfully applied to the study of vertebrate myelinated and non-myelinated axons.

Further improvement in fixation of the squid giant axon has resulted from applying the cannulation method of Adelman and Gilbert (1964) for internal irrigation of the axon with fixative. Acquisition of a Philips EM400 with STEM mode accessories has further broadened the scope of the structural studies and allowed direct comparison of STEM and TEM images of the same specimen area. The use of STEM, particularly with LaB_6 electron sources shows promise of moving towards our goal of better resolution in 3-D with less heavy metal staining.

2) Some progress has been made toward the goal of localizing protein components of the axoplasm by an extension of the giant axon cannulation technique in which the axoplasm is first irrigated with a preparation of the S_1 fraction of myosin prior to intracellular application of fixative.

In order to study the polypeptide composition of axoplasmic filaments, giant axons from the squid L. pealei were carefully dissected in cold, running seawater, cleaned of loose connective tissue, and blotted dry. The axoplasm was gently extruded so as to minimize contamination of the axoplasm by external membrane material and Schwann cells. The axoplasm samples were stored immediately at -70°C . Neurofilaments were structurally intact, and their electrophoretic patterns were unchanged after 12 months of storage.

Identity and general morphology of the neurofilaments during all steps in the purification procedures were monitored by electron microscopy. Axoplasm and neurofilaments were negatively stained with an aqueous solution of 2% uranyl acetate and examined with a JEOL 100C and a Philips EM300 transmission electron microscope.

Protein concentration was determined by the Lowry method. Polyacrylamide gel electrophoresis was carried out by the method of Fairbanks et al., with 5.6% gels. The gels were stained with 0.05% Coomassie blue and scanned with a densitometer from E-C Apparatus. The gels were calibrated with proteins of known size. The 5% Tris/glycine gel system was used to separate the α and β components of tubulin.

Squid axoplasm was prepared for electrophoresis by homogenization in a glass homogenizer with 10 mM Tris·HOAc, pH 7.2/1 mM EDTA. All proteins were treated with 1% sodium dodecyl sulfate and 40 mM dithiothreitol and boiled for 2 min. When the proteolytic inhibitors, phenylmethylsulfonyl fluoride (PMSF, 0.005%) or 1-chloro-4-phenyl-3-tosylamidobutan-2-one (TPCK, 0.001%) were added to homogenized axoplasm, no difference in the gel pattern was observed, suggesting no proteolytic alteration.

Axoplasm was alkylated with iodoacetic acid by the method of Stephens.

Neurofilament enrichment was achieved as follows. Sonication of axoplasm in Tris/EDTA for 5 min was followed by filtration through a Millipore filter. The standard Millipore filtration apparatus attached to a water aspirator was used with a GS filter 4.5 cm in diameter and having a 0.22 μ m pore size.

Axoplasm, electrophoresed in Fairbanks gels, was stained with periodic acid by the Schiff technique in order to determine glycoproteins.

Major Findings.

1) Electron microscopy of the giant axon and smaller fibers of the squid Loligo pealei and of the brain system of the nudibranch Hermisenda crassicornis utilizing improved fixation methods and stereoscopic examination of relatively thick sections (0.2 - 0.5 μ m) has allowed demonstration of a highly ordered neuroplasmic lattice in axons and other neural cellular extensions. The lattice consists primarily of longitudinally oriented neurofilaments and microfilaments, presumably actin, together with microtubules when present, linked together by a well-defined system of thin transverse filamentous bridge elements 2-3 nm in diameter with an apparent periodicity of \sim 40 nm along the axonal longitudinal axis. Internal irrigation of the squid giant axon with fixative following cannulation results in dramatically improved fixation with numerous microtubules being found in the axoplasm, particularly in the subaxolemmal cortical region. The lattice has extensive properties, with domains of order extending over several micrometers. In small axons of both Loligo and Hermisenda, the lattice domain often encompasses the whole fiber diameter. The transverse bridges appear to end at or are structurally continuous with membranous elements such as the axolemma, vesicles, endoplasmic reticulum and mitochondria. It is thought that some or all of the lattice components are involved in axonal transport processes.

The application of a recently acquired Philips EM400 electron microscope with STEM capability has confirmed the characteristics of the neuroplasmic lattice and allowed more precise evaluation of lattice parameters. In particular, the capability of this instrument to compensate for chromatic aberration resulting from the high level of inelastic scattering from thick specimens (1 - 5 μm) in both TEM and STEM mode has allowed the beginning of an evaluation of the long range characteristics of the lattice in relation to spatial distribution within the neuron network and possible correlation with neuronal functions such as transport.

Use of the STEM mode has made possible the observation of structure in sections too lightly stained with heavy metals for the attainment of adequate contrast and resolution in the TEM mode. It is hoped that application of more intense electron sources (such as LaB_6) may further extend the usefulness of this method as part of an overall goal to reduce our dependence on heavy metal staining for adequate contrast and hence resolution of fine structure.

An extension of the STEM technique in which numerous single line scans are signal averaged and subjected to Fourier analysis has allowed independent demonstration of periodic structure in sections of axoplasm where the neuroplasmic lattice order, although difficult to visualize directly, can also be shown by optical autocorrelation techniques.

2) A start has been made towards the goal of identifying and localizing specific neuronal protein components. The internal irrigation fixation technique using cannulations has been modified to allow the introduction of specifically reacting substances such as antibodies into the axoplasm prior to irrigation with fixative. To date, this method has been used to introduce the S_1 fraction derived from rabbit myosin into the squid giant axon in an attempt to "decorate" the presumed actin filaments. Considerable uptake of protein and stabilization of the overall lattice was observed together with some indications of decoration, as yet insufficient to allow precise determination of the f-actin distribution in the axoplasm.

In order to correlate structure with biochemical identification of protein components contributing to these structures, neurofilaments, 10 nm in diameter, from the axoplasm of the squid Loligo pealei, were isolated by a combination of sonication and Millipore filtration. Using this technique, it was shown that actin (43,000 D) and tubulin (56,000 D) are physically separable from intact neurofilaments. The neurofilament-rich retentate contained two major proteins with molecular weights of 200,000 and 63,000.

Publications:

Hodge, A.J. and Adelman, W.J., Jr.: The Neuroplasmic Lattice: Demonstration and Characterization in Loligo and Hermisenda Neurons. J. Ultrastructure Research. 70: 220-241, 1980.

Roslansky, P.F., Cornell-Bell, A., Rice, R.V., and Adelman, W.J., Jr.: Polypeptide Composition of Squid Neurofilaments. Proc. Nat. Acad. Sci. USA. 77: 404-408, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02273-04 LB								
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TITLE OF PROJECT (80 characters or less) An Investigation of Electro-Mechanical Coupled Interaction in Excitable Tissues.										
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SUMMARY OF WRK (200 words or less - underline keywords) The major portion of the research effort was concerned with <u>mechanoelectric transduction mechanisms</u> in squid giant axons. An input-output relationship was observed and present studies will further define and quantitate this relationship.										

Project Description:Objectives.

The isolated squid giant axon preparation offers the opportunity to study excitable membrane electrical responses to various stimuli, including mechanical perturbations. This preparation, which has no known mechanoreceptor function in vivo, is nevertheless particularly attractive in mechanoelectric transducer studies because it lacks the structural complexities which invest most mechanoreceptor organs. Previous work in this laboratory has established the primary electrical response, a depolarization, to a simple stretch (strain) applied to one end of the specimen.

One specific objective in the present study was to characterize the electrical output responses to defined mechanical stimuli. A second objective was to examine input-output phenomena to establish electrical correlates to the various parameters of the mechanical stimulus. A firm establishment of these relationships should more readily render an interpretation of the biophysical processes underlying mechanoelectric transduction.

Methods employed.

A feedback controlled voice coil was used to apply controlled displacements to one end of the axon cylinder. The other end was tied to an in-dwelling glass capillary electrode which was itself carried by a piezoelectric force transducer. This initial configuration was adopted to allow simultaneous recording of the applied mechanical strain and stress in the axon, as well as electrical events. Thus, the membrane area subjected to strain was maximized to permit evaluation of the specific roles of stress and strain in the production of membrane potential responses to mechanical stimuli.

Major findings.

1) Mechanical studies. The most characteristic feature of axon stress following an applied strain is the marked stress relaxation with a time constant of about 1.5 msec or less. Generally, the appearance of stress in the axon was preceded by the initial electrical changes to the applied strain. This observation confirms the suggestion of others that strain is the mechanical entity which initiates the electrical events.

2) Electrical studies. The squid axon preparation shows similarities in the following properties which are characteristic for known mechanoelectric transducer organs. a) Local response. Elongation strain applied to the axon invariably produced membrane depolarization which was not sensitive to TTX, a pharmacological agent which blocks Na^+ channels. The amplitude of this primary response is related to both the amplitude and rate of applied strain. b) The primary response initiates secondary depolarization which is TTX sensitive and can lead to action potential production. The time course of the secondary response is also dependent on the displacement parameters and these relationships are currently under detailed investigation. Finally, a delayed repolarization of the primary response (in the presence of TTX) and the secondary response was observed with displacements of sufficient amplitude and/or duration. Thus, the integrated electrical response to a ramp displacement of specified parameters was a biphasic

signal with depolarization as the primary response to the applied strain.

Proposed Course of Project:

It is proposed to extend this investigation into the following areas:

- 1) Determine the temperature dependence of the primary and secondary responses.
- 2) Obtain a more quantitative assessment of stimulus parameters on output characteristics.
- 3) Evaluate the effects of mechanical stimuli on membrane impedance.
- 4) Extend the inquiry into ionic mechanisms through the use of ionic substitution and pharmacological agents.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02329-03 LB						
PERIOD COVERED October 1, 1979 to September 30, 1980								
TITLE OF PROJECT (80 characters or less) Mechanical properties of resting and stimulated skeletal and/or locomotor muscles.								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: J. B. Wells</td> <td style="width: 33%;">Research Physiologist</td> <td style="width: 33%;">LB NINCDS</td> </tr> <tr> <td>Other: M. Schoenberg</td> <td>Research Physiologist</td> <td>LPB NIAMDD</td> </tr> </table>			PI: J. B. Wells	Research Physiologist	LB NINCDS	Other: M. Schoenberg	Research Physiologist	LPB NIAMDD
PI: J. B. Wells	Research Physiologist	LB NINCDS						
Other: M. Schoenberg	Research Physiologist	LPB NIAMDD						
COOPERATING UNITS (if any) Laboratory of Physical Biology, NIAMDD, Bethesda, MD 20205 Marine Biological Laboratory, Woods Hole, MA 02543								
LAB/BRANCH Laboratory of Biophysics, IRP								
SECTION Section on Neural Membranes (located at MBL, Woods Hole, MA 02543)								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0.0						
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) The effort concerned with excitation-contraction processes in voluntary muscle investigated a possible viscous effect on the instantaneous elastic measurements used to assess cross-bridge activity.								

Project Description:Objectives.

The general objective of this project is to elucidate the chemomechanical transduction processes whereby external mechanical work or tension is produced in activated contractile systems. An earlier achievement of the project was the development of a method to estimate the level of contractile activity in stimulated muscle. The method was based on the measurement of the longitudinal transmission velocity of mechanical impulses in the muscle and a question was recently raised concerning the effect of viscosity on the measurements. The specific objective of a recent series of experiments performed under this project was to examine a muscle preparation throughout activation for evidence of a viscous component.

Methods employed.

Brief, high speed stretches were applied to the preparation and the resulting force production was measured with a high speed piezoelectric force transducer.

Major findings.

A response similar to that recorded from a viscoelastic body was observed during quick stretch of the resting (inactive) muscle. However, the response changed during the activation of the muscle following stimulation and was recognized as a typical response associated with the highly elastic contractile component of active muscle. Thus, what resembled a viscosity in the resting muscle was not confirmed in the active tissue. However, the question concerning the methodology does provoke the suggestion that more work is needed to further confirm the method.

Proposed Course of Project:

It is proposed to extend these studies to in vitro contractile systems to further validate and test the "transmission time" method. Also, the structure of marine invertebrate muscle offers sufficient variation from that of vertebrate muscle to encourage a comparative study of excitation-contraction processes during muscle activation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02151-06 LB
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Information Processing in Simple Nervous Systems.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI.: D.L. Alkon Other: T. Crow I. Lederhendler T. Takeda T. Jerussi A. Kuzirian J. Harrigan J. Neary S. Leighton R. Stephens R. Allen E. Barnes J. Buchanan	Medical Officer Staff Fellow Visiting Fellow Visiting Fellow IPA Fellow Extramural Fellow Mariculturist Biochemist Guest Worker Guest Worker Guest Worker Medical Student Graduate Student	LB NINCDS LB NINCDS LB NINCDS LB NINCDS LB NINCDS LB NINCDS MBL MBL LB NINCDS LB NINCDS LB NINCDS Mt. Sinai Med. Sch. Northeastern U. Cont. page
COOPERATING UNITS (if any) Marine Biological Laboratory, Woods Hole, MA 02543; Mt. Sinai Medical School; Northeastern University; University of Oregon; University of Virginia; Princeton University		
LAB/BRANCH Laboratory of Biophysics, IRP		
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INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 9.0	PROFESSIONAL: 8.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The principle objective is to study the mechanisms by which simple <u>neural networks</u> process information with particular emphasis on mechanisms of <u>learning</u> . The nervous system of <u>Hermissenda crassicornis</u> has proven to be a good model for <u>information processing</u> at several levels: <u>sensory transduction</u> by photoreceptors and hair cells, analysis of <u>synaptic circuitry</u> , changes in synaptic circuitry produced by conditioning paradigms administered to intact animals, as well as to isolated nervous systems, membrane properties modified by <u>conditioning</u> , identification of critical developmental stages for the neural networks of interest, as well as stages critical for learning. Techniques employed thus far to pursue these questions include simultaneous <u>intracellular recording</u> from multiple neural elements, paired stimulation of the visual and vestibular pathways using a rotating table, iontophoresis of fluorescent dyes and electron dense materials, automated <u>behavioral monitoring</u> of intact <u>Hermissenda</u> , <u>voltage-clamp</u> of identified neural elements. Other methods include <u>mariculture</u> , subcellular fractionation, protein phosphorylation analysis, and uptake of neurotransmitter precursors.		

Other Professional Personnel Engaged (continued)

S. Senft	Graduate Student	U. of Oregon
A. West	Graduate Student	U. of Virginia
J. Farley	Guest Worker	LB NINCDS

Project Description:Objectives.

1) To study the mechanisms by which simple neural networks process information with particular emphasis on mechanisms of learning. Information processing at several levels is of interest:

- a. Sensory transduction by photoreceptors and hair cells.
- b. Synaptic interactions between primary sensory receptors.
- c. Synaptic interactions between primary and higher order neural elements.
- d. Intersensory communication: e.g., synaptic interaction between the visual and gravitational sensory pathways.
- e. Changes of synaptic interaction produced by conditioning paradigms administered to the intact animals, as well as to the isolated nervous systems.
- f. Membrane and synaptic properties modified by conditioning.
- g. Identification of critical developmental stages of the neural networks studied, as well as stages critical for learning.
- h. Biochemical mechanisms responsible for long-term neural changes with associative learning.

Methods employed.

1) The nudibranch mollusc Hermissenda crassicornis is the principle experimental preparation. Other marine species, including most recently the lobster, Homarus americanus, are also being screened to provide favorable preparations for specific experimental questions. Intracellular recording from several neural cells simultaneously and voltage-clamp with two microelectrodes in the same cell have been the main techniques used thus far. Means for simultaneously stimulating the chemosensory, visual and vestibular pathways while recording intracellular potentials have been developed in our laboratory. Iontophoresis of fluorescent dyes (e.g., Procion Yellow) and electron dense materials (e.g., cobalt) are also being used extensively.

2) Other methods allow biochemical, electron microscopic and developmental approaches to the problems of interest. These include mariculture, subcellular fractionation, protein phosphorylation analysis, uptake of neurotransmitter precursors, etc. Automated behavioral monitoring permits long-term studies of intact Hermissenda during associative learning.

Major Findings.

Past work has focused on seven major areas:

- a. Behavioral conditioning with neural correlates.
- b. Cellular conditioning in isolated nervous systems.
- c. Neural network analysis.

- d. Receptor physiology.
- e. Voltage clamp of identified neurons.
- f. Biochemistry of Hermisenda neurons.
- g. Neural development.

a,b. Behavioral and cellular conditioning. For the last few years the major focus of the section has been an integrated multidisciplinary effort to determine a neural and a biochemical basis for an associative learning model with the nudibranch mollusc Hermisenda crassicornis. A number of invertebrate species were considered as potential model systems to analyze cellular mechanisms of behavior and learning. These included Tritonia, Aplysia, Pleurobranchia, Helix, Elysia, and Haminoea. The last two have been cultivated within the laboratory and subjected to preliminary electrophysiologic and histologic investigation. The nudibranch mollusc Hermisenda crassicornis, however, has proven to be a most opportune preparation in satisfying the host of constraints which arose from the questions which were asked. With Hermisenda, it has been possible to define a model of associative learning with the same defining features used for vertebrate associative learning. Movement of Hermisenda toward a light source is markedly reduced after repeated pairing of a light stimulus with rotation. This behavioral change is truly associative (i.e., random light and rotation do not produce the effect), persists for at least several days after training and increases with practice. Stimulus specificity for this behavioral change was indicated by the fact that trained animals did not show changes in responsiveness to food. Because of the relative simplicity of the nervous system, it has been possible to ascertain many of the invariant aspects of the three sensory pathways essential to the associative learning model: the visual, statocyst, and chemo-sensory pathways.

Changes have been found (within these neural systems of Hermisenda) which occurred only in animals subjected to associative learning paradigms and not to control paradigms. For example, with the first associative training procedure used, it was found that hair cells received less excitatory input from ipsilateral Type A photoreceptors after repeated stimulus pairing but not after control training paradigms. Comparable neural modification could be produced while recording intracellularly. Thus, it was possible to monitor the neural changes as they were progressively produced by the associative training procedure.

Behavioral analyses of the main experimental animal Hermisenda crassicornis have ranged from field observations to comparative studies of laboratory-reared and collected species. Findings of the previous year, for instance, showed that the light response involves a preference for certain levels of intensity, and a biphasic approach/withdrawal process which depends on an individual animal's light history. This behavior is consistent with the predictions from a recent model of phototaxis which assumes that species have preferences for optimum levels of ambient illumination. Field observations, on the other hand, indicate that natural Hermisenda populations undergo diurnal vertical migrations which are determined not only by ambient light and temperature conditions, but also by food availability.

During the last year, complementary behavioral and neurophysiological studies examined stimulus specificity for associative behavioral changes learned by Hermisenda. Paired, but not random, presentations of a light step with

rotation-produced stimulation of caudal hair cells produced a significant increase in response latency for the animals' movement towards a test light. This associative behavioral change did not occur when cephalic, rather than caudal, hair cells were those stimulated by rotation. Indeed, response latencies were faster for animals receiving paired vs. random presentations of light and cephalic hair cell stimulation and, in some instances, faster than original baseline latencies.

Intracellular recordings from Type B photoreceptors in the isolated nervous system indicated the enhancement of a long-lasting membrane depolarization (LLD) under stimulus presentation regimens paralleling those involved in training of intact animals. A single pairing of light and rotation-produced excitation of caudal hair cells was sufficient to produce an average 3-4 mV depolarization of Type B cells, detectable some 60 sec after the offset of light. Depolarization increased with a second pairing to a cumulative level of 7 mV. Unpaired presentations of light and rotation, as well as light-alone presentations, did not result in an enhanced LLD or a cumulative depolarization. Similarly, paired presentations of light and cephalic hair cell stimulation did not produce an enhanced LLD. Indeed, a slight hyperpolarization (1 mV) was obtained. This sensitivity of cumulative photoreceptor depolarization to the quality of hair cell stimulation derives from the differences in synaptic effects of hair cells upon Type B photoreceptors for caudal vs. cephalic hair cell stimulation. The magnitude and duration of Type B depolarization following light paired with rotation-produced stimulation of caudal hair cells is enhanced by two sources: increased synaptic excitatory input from the ipsilateral optic ganglion and facilitated disinhibition (from caudal hair cells) of the Type B photoreceptor. Turning the circumesophageal nervous system 180° with respect to the center of rotation results in the failure of B cells to receive this synaptic excitation and disinhibition and hence enhancement of the light-induced LLD. This cumulative depolarization specific to stimulus pairing can be expected to result in persistent depolarization (> 3 days) which has been observed for these animals which undergo persistent associative behavioral changes.

Other studies demonstrated that long-term (> 2 days) neural changes in the Type B cell were closely correlated with long-term associative learning of Hermisenda. It was shown that a persistent non-synaptic depolarization of this cell can explain both short- and long-term acquisition and retention of associative behavioral changes. These findings, together with previous neural correlative studies, predicted long-term conductance and biochemical modifications within the Type B cell. These conductances are being comprehensively analyzed with voltage clamp techniques (see below) and their relation to biochemical processes is now being established (see below).

The neural correlate studies have been continued most recently within the motor systems of the visual pathways. Long-term changes of motor neurons (output) specific to associative learning behavior can now be entirely accounted for by differences of Type B input to these cells.

c. Neural network analysis. An understanding of the basis for the cellular and behavioral conditioning previously identified for intact animals, as well as their isolated nervous systems, has been extended within the past year by further study of neural organization for Hermisenda. This study has focused on motor

systems within the sensory pathways. Using intracellular recording and marking techniques, large pedal ganglion motor neurons have been identified within the visual pathway.

Within the past year, impulse activity before, during and after 12 sec light flashes of various intensities was recorded intracellularly from large (200 μm) putative motor neurons in pedal ganglia of semi-intact and isolated nervous system preparations of Hermisenda. LP 1, a cell present only in the left pedal ganglion, responded with significant increases in firing (from 0.4 to 0.5 Hz) in semi-intact animals with or without eyes presented with flashes of light ($10^2 - 10^6 \text{ erg}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$). In the isolated nervous system, however, significant increases were evident with, but not without, the eyes. Moreover, impulse activity of LP 1 was significantly higher in semi-intact than in isolated nervous system preparations with (0.4 vs. 0.1 Hz) or without (0.3 vs. 0.07 Hz) eyes. Significant increases (from 0.7 to 0.9 Hz) were also observed for another left pedal neuron, LP 3, but not for its contralateral homologue, RP 3. Intracellular iontophoresis of fluorescent dye indicated that LP 1 and LP 3 sent efferents to the foot and/or cerata. Stimulating LP 1 caused a brisk movement of the cerata which lasted nearly 2 sec. These results indicate that there are ocular and extraocular inputs to putative motor units in Hermisenda and suggest that the cellular response to light may be lateralized. Moreover, it is possible that ocular input and the subsequent impulse activity may be modulated by the movement of the cerata over the eyes.

Also, during the last year we have developed a new technique for complete reconstruction of individual neurons with conventional microscopic visualization. It is possible to make detailed morphological measurements (i.e., to better than one micron resolution) on the same Hermisenda neuron whose electrical response has been recorded. This is accomplished by impaling a specified neuron, taking electrophysiologic records, and then iontophoresing horseradish peroxidase (HRP) into the neuron. By then passing the tissue through a series of histologic clearing treatments, the surrounding tissue becomes almost transparent while the injected neuron is filled with a light-absorbing reaction product. Using high resolution Differential Interference Contrast microscopy it is possible to selectively image regions of the tissue restricted to a very narrow depth of field. By then incrementing the fine focus control, the entire specimen can be optically sectioned - in each cross section the filled neuron is visually distinct from the surrounding tissue.

Finally, qualitative and quantitative calcium ion studies on the pigment cells and Type B photoreceptors of the adult eye using electron-probe analyses have been undertaken to detect changes in intracellular calcium levels and binding sites in animals experiencing different training paradigms.

d. Receptor physiology. Power spectra of the voltage and current noise in hair cells have been analyzed for a variety of stimulus and treatment conditions. These analyses indicate that the resting conductances of the hair cell membrane are modulated by the rhythmic beating of the cilia. More recently, power spectra of the current noise in hair cells (obtained in voltage-clamped hair cells) have provided more accurate values of hair cell conductances at rest and during stimulation. Under voltage-clamp, the filtering properties of the hair cell membrane no longer distorted the conductance measurements.

Recent voltage-clamp data and current noise analysis support the application of a shot noise model to mechanotransduction by Hermisenda hair cells. We have examined the relaxations of generator currents in voltage-clamped hair cells of Hermisenda crassicornis following brief mechanical displacements. The generator currents were always inward for cells clamped at negative membrane potentials. The generator currents decayed exponentially with a rate constant averaging 0.063 msec^{-1} . The rate constant was voltage-dependent, increasing with hyperpolarization. The power density spectrum of voltage noise observed in these cells during steady state stimulation could be fit with the product of two Lorentzians where τ_1 was similar to the inverse of the rate constant determined from relaxation measurements and τ_2 was the membrane time constant.

In other hair cell experiments during the last year, two complementary approaches were used to immobilize the cilia. Vanadate (+5) anion was iontophoretically injected into hair cells. This reversible inhibitor of dynein cross-bridge cycling initially caused the cilia to lose their normally upright, rigid, vibratile form and assume a more classic, pliable beat pattern. Voltage noise decreased as the cilia showed and bent more extremely, nearly disappearing as motility was lost. When the intracellular vanadate concentration approached 10^{-5} M , the cilia were arrested in an effective stroke against the cell membrane. The cell no longer depolarized upon gravitational or local mechanical stimulation. Rapid reversal of ciliary inhibition with norepinephrine or slow reversal with time restored both the voltage noise and depolarization response. Cilia were rendered rigid and upright by covalent cross-linkage of their membrane "sleeve" to the $9 + 2$ axoneme, using the photoactivated, lipophilic, bifunctional agent 4,4'-dithiobisphenyl azide. In the initial stages of cross-linkage, the cilia remained vibratile but slowed and moved through wider excursions. Voltage noise decreased in frequency but increased in amplitude. When the cilia were fully arrested, voltage noise was minimized while the resting potential and membrane resistance remained essentially constant. Mechanical stimulation of the rigid cilia, normal to the cell membrane, elicited a generator potential of the same amplitude but of greater duration than before treatment. Since cilia which are partially arrested by vanadate undergo increased binding but the hair cell shows decreased noise, neither the axoneme nor the ciliary membrane proper would appear to be sites of direct transduction. In full vanadate arrest, the exposed plasma membrane itself shows no response to stimulation. In cells with beating, stiffened cilia, however, the voltage noise becomes amplified, implying an increased efficiency of transduction. These data indicate that active but rigid flexure of the axoneme near the base of the cilium is required for continuous transduction. The ciliary necklace region is the most likely transduction site, being the terminal leverage point through which force is applied to the membrane via the flexing ciliary shaft.

e. Voltage-clamp of Hermisenda neurons. Experimental results indicated that a primary neural change occurred (with the learning model) within the Type B cells of the Hermisenda eye. Current and voltage-clamp recordings from this cell revealed the presence of a prolonged voltage-dependent Ca^{++} current during and after light steps. The voltage-dependence of this Ca^{++} current and a Ca^{++} -dependent K^+ current provide a neural basis for the contingency necessary to the associative learning model. A voltage-clamp study of the Type B photoreceptor is in progress with the intent of fully characterizing light and voltage-dependent

conductances. Step clamp commands from a holding potential of -60 mV elicit an early, transient outward current that appears to be carried predominantly by K^+ ions. Both steady state activation (peak value) and inactivation of the conductance are voltage-dependent. Activation shows a maximum, "e"-fold change 12.5 mV over the range of -20 to +10 mV. The outward current shows inactivation from a relative value of 1 at -70 mV to zero at about -10 mV. -30 mV is the voltage at which the current is half inactivated at steady state. Unlike some other gastropod neurons in which similar currents have been reported, such as *Anisodoris*, the kinetics of inactivation, like the steady state value, show voltage dependence. The decline of the outward current during sustained depolarization can be fitted by two exponentials, one with a comparatively short time constant which declines from about 130 msec at -20 mV to about 50 msec at +10 mV. The longer time constant (> 1 sec) may reflect the behavior of another outward conductance.

The early outward current is strongly activated at the time that the light-induced sodium current is rising. The temporal position of these two currents suggests that possible extrinsic control of resting potential by paired stimulus presentations could influence the rising phase of the generator potential by setting the degree of early outward current inactivation.

f. Neurochemistry. Recently, we found that the voltage-dependent Ca^{++} and Ca^{++} -dependent K^+ conductances (which provide a contingency mechanisms for the associative learning model) can be regulated by intracellular injection of cyclic-AMP (and not 5'-AMP). This finding suggested biochemical studies (e.g. of protein phosphorylation and protein synthesis) which were conducted during the last year. We have examined protein phosphorylation in the eyes of animals from trained, unpaired, random, and normal control groups. Following three days of behavioral training, the circumesophageal nervous systems were dissected from trained animals and control groups and incubated separately in artificial sea water containing 0.625 mCi ^{32}P i/ml for 2 hr at 15°C. No significant differences were detected in total protein phosphorylation levels (paired $\bar{X}=10,500 \pm 4910$ cpm/eye; random $\bar{X}=10,900 \pm 5410$ cpm/eye; unpaired $\bar{X}=9560 \pm 3320$ cpm/eye). Aliquots of the eyes were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis, and autoradiograms of the gels were analyzed by densitometry. Seven phosphoprotein bands were detected in the eyes from all animals. In paired vs. control groups, there were no significant differences in five of the phosphoprotein bands. However, significant overall differences between paired and control groups ($p < 0.01$) were found for bands with approximate MW of 23,000 and 20,000 daltons. Post-hoc comparisons revealed that the paired group was significantly different from both the random and unpaired control groups ($p < 0.01$), while the controls were not different from each other. One phosphoprotein band (approx. MW=23,000) was increased 49% in paired as compared to random controls. The second phosphoprotein band (approx. MW=20,000) was increased 56% in paired as compared to random controls. The changes in the two phosphoprotein bands, as mediated perhaps by protein kinases and/or phosphatases, may be related to the increase in input resistance in Type B photoreceptors observed in trained animals and to the increase in the long-lasting depolarization in Type B photoreceptors observed in isolated nervous systems following pairing. In summary, the data presented here indicate that the phosphorylation of specific proteins in the eyes is correlated with the acquisition of an associative behavioral modification in Hermisenda.

g. Neural development. Serial electron microscopic sections of developing forms has, during the last year, permitted accurate staging of sensory pathway formation in Hermisenda. Differentiation of neural tissue into the hair cells of the statocyst has been followed from approximately eight days post-fertilization. Formation of photoreceptors, optic ganglion and chemosensory organs was also followed with serial sections. Detailed comparisons with cellular and subcellular specializations of neural elements within these pathways has also been initiated.

Proposed Course of Project.

1) Precise analysis of synaptic interactions between cells within the aforementioned neural networks will be continued with the techniques of intracellular recording and iontophoresis. Particular emphasis will be placed on electron microscopic visualization and reconstruction of cell contacts aided by distribution of hydrogen peroxidase within axons and terminal branches. These studies will not be limited, however, to the networks already discussed. The motor units within the sensory pathways (visual, statocyst, and chemosensory) will be identified. In addition, other more evolved animal forms with potentially analyzable neural networks and behavior will be explored.

2) Anatomic, as well as electrophysiologic, correlates of behavioral and developmental changes will be sought. Using voltage-clamp techniques, cellular mechanisms responsible for the learning model will be further analyzed. Regulation of potential-dependent currents believed to, at least in part, underlie the observed behavioral changes will be given particular attention.

3) Biochemical and pharmacologic analyses of relevant neural systems will continue. We will continue to study subcellular and/or biochemical loci at which primary behaviorally-meaningful changes occur. The Type B photoreceptor will be the initial focus of this work.

We plan to identify the mechanisms leading to the observed changes in protein phosphorylation specific to learning in Hermisenda, i.e., involving changes of cyclase, phosphodiesterase, protein kinase or phosphatase activities. It may also be possible to detect changes in levels of cyclic nucleotides by immunocytochemistry. Initial experiments have utilized isolated nervous systems in order to establish the biochemical detection procedures in our laboratory, but more recent studies involve analysis of phosphorylation in individual Hermisenda neurons.

Other mechanisms of post-translational modification will also be explored. Use of the high resolution, two-dimensional method of O'Farrell is planned to determine if specific proteins are methylated or demethylated during associative training.

We also will study the synthesis and modification of gene products in the Hermisenda nervous system by means of high resolution, two-dimensional electrophoresis. O'Farrell has shown that the 50 or so protein bands visible on conventional one-dimensional gels can be resolved into 1100 proteins by combining isoelectric focusing in the first dimension with SDS-slab gel electrophoresis in the second dimension. A modification in the basic O'Farrell technique provides for the separation of nuclear proteins.

4) Behavioral experiments will be continued to further determine the comparability of the Hermisenda associative learning model to associative learning defined for more evolved species.

We will continue ongoing time-lapse studies of behavior so that motor activity patterns and response to food, cover and conspecifics of wild, laboratory-reared, and experimentally manipulated animals can be described under a variety of illumination conditions. To aid in the analysis of the data generated by this approach, a digitizer interfaced with a computer will be used to record the data directly on tape and then analyze it. The relationship of laboratory to natural habitat behaviors will also be pursued by making additional observations of Hermisenda in the wild.

5) The generality of cellular principles of learning and development determined for relatively "simple" neural systems and the behavior they control will be examined. Ultimately, mechanisms common to organisms with a wide range of evolutionary diversity and complexity may contribute to understanding the human nervous system and may motivate clinical approaches.

Publications:

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Akaike, T. and Alkon, D.L.: Sensory convergence on central visual neurons in Hermisenda. J. Neurophysiol. 44: 501-513, 1980.

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Stommel, E.W., Stephens, R.E., and Alkon, D.L.: Motile statocyst cilia transmit rather than transduce mechanical stimuli. J. Cell Biol. (In press).

Farley, J. and Alkon, D.L.: Neural organization predicts stimulus specificity for a retained associative behavioral change. Science. (In press).

Alkon, D.L.: Membrane depolarization accumulates during acquisition of an associative behavioral change. Science. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02088-07 LB																											
PERIOD COVERED October 1, 1979 to September 30, 1980																													
TITLE OF PROJECT (80 characters or less) Function and Structure of Membrane Ionic Channels: Pharmacology and Ionic Selectivity																													
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<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">G. Ehrenstein</td> <td style="width: 40%;">Research Physicist</td> <td style="width: 20%;">LB NINCDS</td> </tr> <tr> <td rowspan="6">Other:</td> <td>L. Binstock</td> <td>Electronic Engineer</td> <td>LB NINCDS</td> </tr> <tr> <td>R.E. Taylor</td> <td>Research Physiologist</td> <td>LB NINCDS</td> </tr> <tr> <td>F. Bezanilla</td> <td>Professor</td> <td>UCLA</td> </tr> <tr> <td>L.M. Huang</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>B.S. Wong</td> <td>Visiting Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>H. Lecar</td> <td>Research Physicist</td> <td>LB NINCDS</td> </tr> <tr> <td></td> <td>W.A. Catterall</td> <td>Professor</td> <td>Univ. of Wash.</td> </tr> </table>			PI:	G. Ehrenstein	Research Physicist	LB NINCDS	Other:	L. Binstock	Electronic Engineer	LB NINCDS	R.E. Taylor	Research Physiologist	LB NINCDS	F. Bezanilla	Professor	UCLA	L.M. Huang	Staff Fellow	LB NINCDS	B.S. Wong	Visiting Fellow	LB NINCDS	H. Lecar	Research Physicist	LB NINCDS		W.A. Catterall	Professor	Univ. of Wash.
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	H. Lecar	Research Physicist	LB NINCDS																										
	W.A. Catterall	Professor	Univ. of Wash.																										
COOPERATING UNITS (if any) Marine Biological Laboratory, Woods Hole, Massachusetts UCLA School of Medicine, Los Angeles, California University of Washington, Seattle, Washington																													
LAB/BRANCH Laboratory of Biophysics																													
SECTION Section on Molecular Biophysics																													
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																													
TOTAL MANYEARS: <div style="text-align: center;">4.6</div>	PROFESSIONAL: <div style="text-align: center;">3.8</div>	OTHER: <div style="text-align: center;">0.8</div>																											
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINDRS <input type="checkbox"/> (a2) INTERVIEWS																													
SUMMARY OF WORK (200 words or less - underline keywords) <p>The long-range purpose of this project is to study the function and structure of <u>membrane ionic channels</u> and their interaction with drugs.</p> <p>We have determined the effects of <u>tetraethylammonium (TEA)</u> and <u>quinidine</u> on the <u>Myxicola</u> giant axon. TEA works on both the internal and external membrane surfaces. The primary effect of quinidine is to block potassium channels, and this action may explain its usefulness as an <u>antiarrhythmic agent</u>.</p> <p>We have also determined the effects of <u>tetrodotoxin</u> and <u>local anesthetics</u> on tissue-cultured cells. Charged and uncharged local anesthetics act at different sites in the sodium channel.</p>																													

Project Description

Objectives: To determine the mechanism of action of drugs on membrane ionic channels. Particular emphasis is on drugs that are clinically useful and drugs that are useful in studying the ionic channels. To study channel properties by means of gating current measurements.

Methods Employed: For experiments on the Myxicola axon, a standard giant axon voltage clamp is used. For experiments on tissue-cultured cells, two methods are used to measure currents through ionic channels. In one method, channels are kept open by means of channel-opening drugs, and radioactive uptake is used to measure ion flux. In the other method, which we are now developing, the small tissue-cultured cells are voltage clamped using an approach recently developed by Kryshchal and Pidplichko. This involves puncturing the cell with an electrode, and allowing it to reseal. The puncturing electrode is then used for both electrical and chemical access to the cell interior.

For gating current measurements, a voltage clamp is used together with several specialized circuits. These allow measurement of the current difference between positive and negative pulses in order to eliminate ordinary capacitive current. Ionic currents are eliminated by use of drugs, notably tetrodotoxin. As a result of these procedures, the gating current can be separated and recorded.

Major Findings: Recent gating current measurements have concentrated on slow sodium inactivation, and we are attempting to incorporate this new information into a general model that will explain all of the gating current information.

The TEA experiments on Myxicola have shown that there are sites on the external membrane surface where TEA can act. This is something of a surprise, since it means that in this regard Myxicola is much more like frog node than like another giant axon - squid.

The quinidine experiments on Myxicola have shown a number of effects, but the largest effect was clearly a reduction in the potassium current. This effect has been carefully studied. Quinidine blocks both inward and outward potassium currents and blocks less well in the presence of high external potassium concentration. These results have been incorporated into a model for quinidine action, which requires further testing. These quinidine effects could explain the efficacy of this drug as an antiarrhythmic agent.

Experiments on neuroblastoma cells have been made with charged local anesthetics, with uncharged local anesthetics, and with both types present simultaneously. A comparison of the dose-response curves for the three cases showed that both charged and uncharged local anesthetics block sodium channels, but that they do so from different sites. This means that mixtures of charged and uncharged local anesthetics would have increased efficacy. We are now contacting clinical researchers to determine whether local anesthetic combinations might be useful in reducing dosages, and hence side effects.

Publications:

Huang, L.M., Catterall, W.A. and Ehrenstein, G.: Comparison of ionic selectivity of batrachotoxin-activated channels with different tetrodotoxin dissociation constants. J. Gen. Physiol. 73: 839-854, 1979.

Wong, B.S: Quinidine interactions with Myxicola giant axons. Biophys. J. (In press).

Taylor, R.E. and Bezanilla, F.: Comments on the measurement of gating currents in the frequency domain. Biophys. J. 26: 338-340, 1979.

Taylor, R.E, Bezanilla, F. and Rojas, E.: Diffusion models for the squid axon Schwann cell layer. Biophys. J. 29: 95-117, 1980.

Project Description

In collaboration with S.I. Rapoport and others, a compartmental model has been proposed for the entry of drugs into the brain and cerebrospinal fluid, to explain the time course of their distribution there. Good fits were found to data from experiments using radioactive urea as the solute.

Work has continued on a generalization of D. G. Levitt's model for an ion in a cylindrical pore in a dielectric membrane. Several different numerical methods of computation have been tested. Contour plots of potential levels in and around the membrane have been produced to help in visualizing the physical phenomenon. A computer program incorporating fixed charges in the pore surface is being tested for use in comparing experimental results on membrane pores with the model.

The experiments by Dr. W. J. Adelman and Dr. Louis DeFelice on the sinusoidal current clamp produce components of current at three times as well as twice the fundamental frequency. Equations for calculating these third-order currents for the Hodgkin-Huxley model have been derived, and computations made which show qualitative agreement with experiments.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER <p style="text-align: center;">Z01 NS 02218-05 LB</p>																	
PERIOD COVERED October 1, 1979 to September 30, 1980																			
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Voltage-Dependent Ionic Conductance in Membranes</p>																			
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																			
<table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">D.L. Gilbert</td> <td style="width: 35%;">Research Physiologist</td> <td style="width: 15%;">LB NINCDS</td> </tr> <tr> <td rowspan="4">Other:</td> <td>G. Ehrenstein</td> <td>Research Physicist</td> <td>LB NINCDS</td> </tr> <tr> <td>L.M. Huang</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>H. Lecar</td> <td>Research Physicist</td> <td>LB NINCDS</td> </tr> <tr> <td>C. Morris</td> <td>Postdoctoral Fellow</td> <td>LB NINCDS</td> </tr> </table>			PI:	D.L. Gilbert	Research Physiologist	LB NINCDS	Other:	G. Ehrenstein	Research Physicist	LB NINCDS	L.M. Huang	Staff Fellow	LB NINCDS	H. Lecar	Research Physicist	LB NINCDS	C. Morris	Postdoctoral Fellow	LB NINCDS
PI:	D.L. Gilbert	Research Physiologist	LB NINCDS																
Other:	G. Ehrenstein	Research Physicist	LB NINCDS																
	L.M. Huang	Staff Fellow	LB NINCDS																
	H. Lecar	Research Physicist	LB NINCDS																
	C. Morris	Postdoctoral Fellow	LB NINCDS																
COOPERATING UNITS (if any) <p>R.J. Lipicky, Food and Drug Administration E. Wenkert, Chairman, Dept. of Chemistry, Rice University, Texas</p>																			
LAB/BRANCH Laboratory of Biophysics																			
SECTION Section on Molecular Biophysics																			
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																			
TOTAL MANYEARS: <p style="text-align: center;">1.9</p>	PROFESSIONAL: <p style="text-align: center;">1.4</p>	OTHER: <p style="text-align: center;">0.5</p>																	
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div style="width: 30%;"> <input type="checkbox"/> (c) NEITHER </div> </div>																			
SUMMARY OF WORK (200 words or less - underline keywords)																			
<p>One goal of this project is to better understand the mechanisms of the <u>ionic conductance in membranes</u> which are voltage-dependent and excitable. Another goal is to determine how <u>drugs</u> influence these channels. These studies involve the use of the <u>squid giant axon</u> and the <u>giant barnacle muscle fiber</u>. We have continued studies on the mechanism of drug-channel interactions in the <u>squid giant axon membrane</u>. In particular, we have studied <u>yohimbine</u>, <u>phenytoin</u>, and <u>perhexiline</u>, and found that all three exhibit voltage-dependent behavior. We have also studied different types of oscillatory behavior in the barnacle muscle fiber, and developed a mathematical model to explain the behavior. The model is based on experimentally-determined properties of potassium channels and calcium channels.</p>																			

Project Description

Objectives: To determine the properties of voltage-dependent channels. To determine the mechanisms for interactions of these channels with drugs. To characterize oscillatory behavior in axons and to relate this behavior to the properties of ionic channels.

Methods Employed: The primary methods employed in this project are voltage-clamping and current-clamping.

Major Findings: Yohimbine exhibits both a use-dependent and a tonic inhibition of the sodium currents. We are at present studying the structure-activity relationship of various yohimbine analogs, specifically synthesized for this purpose. We have tested cis-trans isomers of the various groups and have found that β yohimbine and yohimbyl alcohol exhibit the same approximate quantitative effects as yohimbine does. Two other drugs also exhibit use-dependent inhibition. They are phenytoin, an anti-arrhythmic drug, and perhexiline, an anti-anginal drug. The dose-response curve of phenytoin was shifted to the right when the membrane voltage was held at a higher potential, i.e., the sodium currents increased for the same phenytoin concentration when the membrane was hyperpolarized. In contrast to the yohimbine use-dependence, the use-dependent inhibition by phenytoin increased significantly when the depolarizing pulse duration was increased. The action of perhexiline has been theorized to be due to its calcium antagonism. Calcium shifts the h -infinity versus potential relation along the potential axis. Perhexiline produces no such shift. Therefore, the action of perhexiline is probably not due to calcium antagonism. Perhexiline seems to work close to the internal membrane, since its action is rapid when internally perfused and is extremely slow when externally applied.

Barnacle muscle fibers subjected to constant current stimulation produce a variety of types of oscillatory behavior when the internal medium contains the Ca^{++} chelator EGTA. Oscillations are abolished if Ca^{++} is removed from the external medium, or if the K^+ conductance is blocked. Available voltage clamp data indicate that the cell's active conductance systems are exceptionally simple. Given the great complexity of barnacle fiber voltage behavior, this seems paradoxical. However, our analysis of the possible modes of behavior available to a system of two non-inactivating conductance mechanisms, indicates a good correspondence with the types of behavior exhibited by barnacle fiber in its bistable and primary oscillation modes. The differential equations of a simple equivalent circuit for the fiber are dealt with using some of the mathematical techniques of non-linear mechanics. General features of the system are a propensity to produce damped or sustained oscillations over a rather broad parameter range, and considerable latitude in the shape of the oscillatory potentials. It is concluded that for cells subject to changeable parameters (either from cell to cell or with time during cellular activity) a system dominated by two non-inactivating conductances could be the source of a rich repertoire of voltage behavior.

Publications: Gilbert, D.L.: Discussion: What controls atmospheric oxygen? BioSystems 12: 123-124, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02219-05 LB														
PERIOD COVERED October 1, 1979 to September 30, 1980																
TITLE OF PROJECT (80 characters or less) Structure and function of the perineurium																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">R.E. Taylor</td> <td style="width: 40%;">Research Physiologist</td> <td style="width: 10%;">LB NINCDS</td> </tr> <tr> <td rowspan="3">Other:</td> <td>S.I. Rapoport</td> <td>Medical Officer, Researcher</td> <td>LN NIA</td> </tr> <tr> <td>A. Weerasuriya</td> <td>Visiting Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>N. Shinowara</td> <td>Staff Fellow</td> <td>LN NIA</td> </tr> </table>			PI:	R.E. Taylor	Research Physiologist	LB NINCDS	Other:	S.I. Rapoport	Medical Officer, Researcher	LN NIA	A. Weerasuriya	Visiting Fellow	LB NINCDS	N. Shinowara	Staff Fellow	LN NIA
PI:	R.E. Taylor	Research Physiologist	LB NINCDS													
Other:	S.I. Rapoport	Medical Officer, Researcher	LN NIA													
	A. Weerasuriya	Visiting Fellow	LB NINCDS													
	N. Shinowara	Staff Fellow	LN NIA													
COOPERATING UNITS (if any) Laboratory of Neurosciences, NIA																
LAB/BRANCH Laboratory of Biophysics																
SECTION Section on Molecular Biophysics																
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																
TOTAL MANYEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">0.8</div>	OTHER: <div style="text-align: center;">0.3</div>														
CHECK APPROPRIATE BOX(ES)																
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																
SUMMARY OF WORK (200 words or less - underline keywords)																
<p>The purpose of this project is to determine how and to what extent the perineurium is involved in the maintenance and regulation of the ionic and metabolic environment of the axons of peripheral nerves. The topics of interest include: (1) <u>passive permeability</u> to electrolytes and nonelectrolytes; (2) <u>active transport</u> of ions; (3) facilitated diffusion or transport of amino acids and glucose; (4) electrical impedance and short circuit currents; (5) the determination of the normally existing composition of the <u>extracellular fluid</u> in the <u>endoneurium</u>.</p>																

Project Description:

The extracellular space in the endoneurium of peripheral nerve is isolated by the endothelial lining of cell capillaries and by the simple layer of cells in the perineurium which are connected together by tight junctions. This project is concerned with the study of the role of the epithelial cell layer in the perineurium.

The methods employed are principally the standard techniques used to study unidirectional fluxes of various substances across the isolated and perfused perineural sheath of the frog or toad, including the use of radioactive tracers. In addition, histological techniques are employed using electron microscopy, and electrical measurements are made using internal and external voltage and current supplying electrodes.

Permeation rates for both sodium and potassium were found to be the same in either direction across the perineurium and were unaffected by metabolic inhibitors. The mean value measured for the sodium permeability coefficient was 1.7×10^{-6} cm/sec. There is no discrimination between potassium and chlorine ions, but the permeability of potassium exceeds that of sodium by more than would be predicted by free solution mobilities.

It is concluded that there is no evidence for active sodium or potassium transport across the perineurium, and that the paracellular path in the perineurium exhibits size-dependent permselectivity properties. Furthermore, the low rates of transperineurial permeation of ions and water-soluble non-electrolytes are comparable to those in epithelia with tight junctions.

We have successfully completed work on the changes in permeability induced by Wallerian degeneration and recovery and on the development of the rat perineurium preparation.

Publications:

Weerasuriya, A., Rapoport, S.I. and Taylor, R.E.: Modification of permeability of frog perineurium to ^{14}C -sucrose by stretch and hypertonicity. Brain Research 173: 503-512, 1979.

Weerasuriya, A., Rapoport, S.I. and Taylor, R.E.: Ionic permeabilities of the frog perineurium. Brain Research 191: 405-415, 1980.

Weerasuriya, A., Rapoport, S.I. and Taylor, R.E.: Perineurial permeability increases during Wallerian degeneration. Brain Research 192: 581-585, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02316-03 LB
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Comparison of Different Modes of Axonal Stimulation		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; margin-top: 100px;"> <div>PI: G. Ehrenstein</div> <div>Research Physicist</div> <div>LB NINCDS</div> </div>		
COOPERATING UNITS (if any) G. Ganot, Technion Medical School, Haifa, Israel		
LAB/BRANCH Laboratory of Biophysics, IRP		
SECTION Section on Molecular Biophysics		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.6</div>	PROFESSIONAL: <div style="text-align: center;">0.3</div>	OTHER: <div style="text-align: center;">0.3</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p style="text-align: center;"> There are differences in the response of <u>excitable membranes</u> to different modes of stimulation. For example, <u>sodium channels</u> inactivate following electrical stimulation, but do not inactivate following chemical stimulation (by batrachotoxin, for example). The goal of this project is to compare and contrast the responses of ionic channels to electrical, chemical, and <u>mechanical stimulation</u>. </p>		

Project Description

Objectives: To determine the mechanism for mechanical transduction in axons, and to compare this mechanical transduction in other tissues. To compare the responses of ionic channels to electrical, chemical, and mechanical stimulation.

Methods Employed: A giant axon was mounted horizontally in a standard voltage clamp chamber. Mechanical stimuli were supplied to the axon from above by the movement of a loudspeaker driven by a power amplifier and controlled by a pulse generator. The loudspeaker was coupled to the axon by means of a thin metal rod connected to a plastic stylus 2 mm in diameter. This assembly was positioned just above the voltage sensor of the internal electrode of the voltage clamp. Movement of the stylus interrupted the signal between a light-emitting diode (LED) and a photoresistor so that the output of the photoresistor monitored the position of the stylus. The rise time for the stylus movement was about 1 msec.

Major Findings: Several different types of axons tested responded to mechanical stimulation in qualitatively similar ways. For experimental convenience, our detailed studies have been on the Myxicola giant axon. We obtained current-voltage curves for a given amplitude of mechanical stimulation by clamping the axon to each desired voltage, waiting until the current reached steady state, applying the mechanical stimulus, and then recording the new steady state current. For a relatively small amplitude of stimulation, the reversal potential was about -40 mV, and for large amplitudes of stimulation, it was about 0 mV.

There are two simple models that could explain the observed change in reversal potential. In the first model, there are at least two different types of channels with different reversal potentials, and one or another type is preferentially excited, depending upon stimulus amplitude. In the second model, there is only one type of "channel", but its effective diameter increases with increasing stimulus amplitude. For example, the channel may be somewhat selective to potassium for small mechanical stimuli, but rather unselective for large mechanical stimuli.

According to the first model, the current record during voltage clamp should contain at least two different time courses, corresponding to the different channels present. Experimentally, the current records show only a single time course. Thus, the second model, with a channel diameter that gradually increases as the amplitude of the mechanical stimulus increases, seems to be the correct one.

Proposed Course of Project: To further test the model proposed above, we plan to investigate in more detail the selectivity properties of the mechanically stimulated axon. We plan to measure the effect of changes in external ionic environment on membrane current, and make a more complete determination of the dependence of the reversal potential on the amplitude of the mechanical stimulus.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER <div style="text-align: right;">Z01 NS 02317-03 LB</div>																										
PERIOD COVERED <div style="text-align: center;">October 1, 1979 to September 30, 1980</div>																												
TITLE OF PROJECT (80 characters or less) <div style="text-align: center;">Excitable Membranes of Tissue-cultured Nerve and Muscle Cells</div>																												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 35%;">H. Lecar</td> <td style="width: 40%;">Research Physicist</td> <td style="width: 15%;">LB NINCDS</td> </tr> <tr> <td rowspan="7">Other:</td> <td>M. Jackson</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>C. Morris</td> <td>Postdoctoral Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>L. M. Huang</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>N. Moran</td> <td>Postdoctoral Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td>G. Ehrenstein</td> <td>Research Physicist</td> <td>LB NINCDS</td> </tr> <tr> <td>C. N. Christian</td> <td>Staff Fellow</td> <td>LDN NICHD</td> </tr> <tr> <td>C. L. Stephens</td> <td>Staff Fellow</td> <td>I NCI</td> </tr> </table>			PI:	H. Lecar	Research Physicist	LB NINCDS	Other:	M. Jackson	Staff Fellow	LB NINCDS	C. Morris	Postdoctoral Fellow	LB NINCDS	L. M. Huang	Staff Fellow	LB NINCDS	N. Moran	Postdoctoral Fellow	LB NINCDS	G. Ehrenstein	Research Physicist	LB NINCDS	C. N. Christian	Staff Fellow	LDN NICHD	C. L. Stephens	Staff Fellow	I NCI
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COOPERATING UNITS (if any) Medical Neurology Branch, NINCDS; Laboratory of Developmental Neurobiology, NICHD; Immunology Branch, NCI; Laboratory of Biochemical Genetics, NHLBI																												
LAB/BRANCH Laboratory of Biophysics																												
SECTION Section on Molecular Biophysics																												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																												
TOTAL MANYEARS: <div style="text-align: center;">4.2</div>	PROFESSIONAL: <div style="text-align: center;">3.4</div>	OTHER: <div style="text-align: center;">0.8</div>																										
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>																												
SUMMARY OF WORK (200 words or less - underline keywords) <div style="text-align: center;"> <p> <u>Ion channel</u> properties are studied in the <u>excitable membranes</u> of nerve and muscle cells grown in <u>tissue culture</u>. Measurements of <u>single-channel fluctuations</u> are done using an external patch electrode. Such measurements characterize the <u>unit conductance</u> and <u>gating kinetics</u> of the channels. <u>Membrane noise analysis</u>, <u>voltage clamp</u>, and <u>radioactive tracer</u> experiments are used to characterize the gating dynamics, ion selectivity and pharmacological properties of postsynaptic, electrically excitable and immunologically-induced ionic channels. </p> </div>																												

Project Description:

Objectives: To determine the unit conductances and gating dynamics of postsynaptic ionic channels by measurement of agonist-induced electric current fluctuations. To observe ionic-channel complexes induced by complement in antibody-antigen treated cells. To assess the evolution of heterogeneous populations of postsynaptic channels in developing muscle cells. To measure the gating kinetics and conductances of pharmacologically modified ionic channels. To relate the transport properties of the channels to theoretical predictions based on molecular structural information.

Methods Employed: An electrically isolated patch recording system is used to measure extracellular currents at the picoampere level. The isolated patch system is capable of measuring current fluctuations caused by the activation of individual ion-conducting channels within the membrane. Electrical noise analysis, microelectrode voltage clamp recording, and radioactive tracers are used for the study of channel selectivity. Theoretical analysis of gating kinetics, channel transport and membrane noise continue. An internal dialysis method for isolating the membrane of a neuroblastoma cell by sealing the cell to the mouth of a penetrating pipette is under development.

Major Findings: Single channel currents have now been studied extensively for the following systems: cholinergic postsynaptic channels of rat and chick myotubes; complement-induced channels in antibody-antigen treated muscle cells; postsynaptic channels in cultured human skeletal muscle. In addition, the patch electrode method has been tried on a number of preparations to be used in future studies, such as tissue-cultured mouse CNS neurons, in which we have detected glutamate channels, neuroblastoma hybrid cells, which show serotonin-induced electrical activity, macrophage cells, to be used as models for chemoreceptive transduction, and dorsal-root ganglion cells in early developmental stages.

The studies determining the single-cholinergic channel conductances and open-state lifetimes have now been published. Work continues on the use of the patch method to characterize the distribution of junctional and extrajunctional cholinergic receptors in developing rat myotubes. Experiments done to date show evidence for fast and slow channels in rat muscle, but no such distinction in avian muscle. These studies are continuing in collaboration with C. Christian (LDN-NICHD).

Complement-induced channels in antibody-antigen treated muscle cells show a unit conductance of 90 pS and appear to flicker open and closed transiently. The kinetics of the channel-formation, as studied with our technique, appear complicated and variable. The conductance-value we have measured is consistent with the value expected from the single-hit theory of cell-killing by colloid osmotic lysis. A publication has been prepared showing the main features of the transient bursts of channel formation during complement assault of a small patch. These experiments are being done in collaboration with Dr. C.L. Stephens (I NCI), and provide direct observational evidence for self-healing complement lesions which she has studied by other means.

Cholinergic channels were observed in human muscle cells grown in tissue culture by Dr. W.K. Engel and Dr. V. Askanas. The observations to date show a channel conductance of 47 pS, close to the value we have determined for other mammalian skeletal muscle. Preliminary measurements indicate a substantial fraction of long-lived channels with mean open times as long as 20 msec. Such slow channels have been reported by other workers on the basis of electrical noise experiments. Present experiments focus on the precision measurement of the channel lifetimes in cultured human muscle grown according to uniform procedures.

Preliminary experiments on serotonin sensitivity in neuroblastoma-glioma hybrid cells have shown rapid responses usually associated with postsynaptic activity. Serotonin-induced current noise and scattered single-channel events have been observed, but the results on this preparation are still variable.

Significance to Biomedical Research: Ionic channels are the basic units of nerve excitation. The variety of excitable-cell behavior can be understood largely in terms of the properties of a few prototypical channels and their distribution in the cell membrane. Experimental study of ionic channels in tissue-cultured excitable cells allows the characterization of the unit channels by experimental means which do not have to be designed anew for each new type of membrane.

The focus in both the cholinergic channel and complement channel experiments has been on characterizing the kinetics of the conformation change whereby the channel switches from conducting to nonconducting states. In the case of the cholinergic channels, such evidence appears at present to be identifying heterogeneous populations of channels which occur at different stages of the development of excitability. In the case of the complement channel, the data provide direct identification of a pore-like state, which may be an early stage in the complement cascade. The 5HT and other studies with new agonists provide a characterization of the various types of agonist-induced conductance elements in CNS cells which is independent of conditions of cell geometry and state of development. Such information, defining the elementary units of excitability, should effect a considerable simplification for neurophysiological and pharmacological studies of CNS cells.

Proposed course of project: The main thrust of the patch electrode experiments will be to extend the method to neuroblastoma and tissue-cultured CNS neurons. Single-channel recording will be used in conjunction with electrical noise measurements to characterize a number of postsynaptic channels. The patch electrode will also be used to record activity from small cells and in situations where the cell surface can be probed for specific agonist activity.

Publications: Jackson, M.B. and Lecar, H.: Single postsynaptic channel currents in tissue-cultured muscle. Nature 282: 863-864, 1979.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Experimental Neurology
National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report

October 1, 1979 through September 30, 1980

Laboratory of Experimental Neurology, Intramural Research Program
National Institute of Neurological and Communicative Disorders and Stroke

William F. Caveness, M. D.

General Statement:

The Laboratory was established within the Intramural Program, NINCDS, NIH, April 1969. Its scientific objectives have been: to provide fresh insight into the propagation of focal motor seizures in the monkey; to provide precise observations of the delayed effects of ionizing radiation, including therapeutic exposures on the monkey brain; and to uncover the anatomical as well as functional sequelae of craniocerebral trauma in man.

Current Scientific Effort:

1) EXPERIMENTAL FOCAL SEIZURES IN THE MONKEY

The conceptual framework is that the propagation of a focal motor seizure is a highly organized process involving, perhaps in an excessive manner, elements in the sensorimotor system. Attention is directed to relatively large neuronal aggregates, connected by macro-circuitry.

To gain a global appraisal of neuronal activity in cortical and subcortical structures we have employed the [^{14}C] Deoxyglucose method for the determination of regional cerebral glucose utilization.

The seizures were induced by stereotactically controlled injection of 25,000 units of crystalline potassium penicillin G, in 0.025 ml of distilled water, into area 4 of the face-hand area of the right motor cortex at a depth of 2.5 mm.

Bipolar electroencephalographic (EEG) recording was obtained with six Beckman scalp electrodes arrayed bilaterally in three symmetrical pairs over the frontal, temporal, and occipital regions, respectively. Eight pairs of needle electrodes for electromyographic (EMG) recording were placed in the right and left masseter and orbicularis oris muscles and the flexors of the four extremities.

Following the intracortical penicillin injection and after the development of ipsilateral electrographic spikes and the beginning of the contralateral clinical expression, [^{14}C] labeled deoxyglucose, 100 $\mu\text{Ci/kg}$, was injected by vein. After thirty minutes, during which timed

arterial blood samples were obtained, the animal was decapitated, and the head immediately immersed in Freon chilled by liquid nitrogen to -100°C . Subsequently the brain in the skull, was serially sectioned at $30\text{ }\mu\text{m}$ with a PMV cryo-microtome at a temperature of -20°C .

Approximately 100 sections from each brain were dried at 60°C and placed on blue sensitive x-ray film for macroautoradiographs. From the determination of the concentration of $[^{14}\text{C}]$ deoxyglucose and glucose in arterial plasma and the concentration of $[^{14}\text{C}]$ in the autoradiographs, the actual glucose utilization was calculated for 48 bilateral structures within and outside the sensorimotor system.

Yield to date:

Three manuscripts have been published this year in the Annals of Neurology that represent the culmination of the studies on focal motor seizures in the Macaca mulatta. These may be summarized as follows.

LOCAL CEREBRAL GLUCOSE UTILIZATION IN NEWBORN AND PUBESCENT MONKEYS DURING FOCAL MOTOR SEIZURES

ABSTRACT

The objective was to determine differences in the rate of local cerebral glucose utilization (LCGU) in newborn and pubescent monkeys during focal motor seizures. For the controls, 3 newborn and 4 pubescent, and for the seizures, 3 newborn and 6 pubescent monkeys were used. In the controls, the pattern of glucose utilization within the structures of the sensorimotor system was quite different at the two age levels, with the newborn showing far less activity, the most evident difference being in the neocortex and striatum. In the seizure monkeys, the unilateral increase in LCGU relative to the controls was greater in the newborn than in the pubescent monkeys, except in the cerebral and cerebellar cortices. The increased utilization in the cortical and subcortical structures was ipsilateral to the discharging lesion and without the well defined pattern seen in the pubescent monkeys. In general, the newborn was capable of supporting a focal motor seizure but lacked the precise clinical and electrographic expressions or efficient energy metabolism that accompany the maturation of the brain at puberty.

PROPAGATION OF FOCAL MOTOR SEIZURES IN THE PUBESCENT MONKEY

ABSTRACT

The rate of local cerebral glucose utilization was employed for the quantification of energy metabolism in macrostructures of the sensorimotor system during the propagation of focal motor seizures in the 24 month old monkey. This rate was determined in four control monkeys in four with the seizures limited to the contralateral face, in four with the

seizures extending to the contralateral face and upper extremity, and in four in which there was a bilateral expression. There was a sequential increase in glucose utilization, primarily unilateral, with propagation. This was greatest, in order, in the sensory and motor cerebral cortices, putamen and globus pallidus, with somewhat less increase in sensory and motor thalamic relay nuclei, and least in the cerebellar cortex. It was concluded that the increased rate of glucose utilization in the indicated distribution was for the transmission and restraint of the paroxysmal activity, and the essential maintenance of energy equilibrium.

EFFECTS OF MANIPULATION OF THE SENSORIMOTOR SYSTEM ON FOCAL MOTOR SEIZURES IN THE MONKEY

ABSTRACT

During focal motor seizures, induced by injecting penicillin into the face-hand area of the right motor cortex of 24 month old monkeys, the manipulation of the sensorimotor system was brought about by: a) the elimination, through a paralytic agent, of the proprioceptive input from contracting muscles and joints. This caused no significant alteration in the electrographic expression of the seizure and no alteration in the pattern of local glucose utilization in cortical or subcortical components of the sensorimotor system. There was however, an overall increase in the rate of energy metabolism in the paralyzed monkeys with electrographic seizures. These observations respectively underscore the strength of the integrated seizure activity, unaltered in pattern by the removal of a component of the sensorimotor system, and suggests an imbalance in excitation-inhibition mechanisms in the absence of proprioceptive input. b) The cryogenic destruction of up to 90% of the ipsilateral ventral caudal globus pallidus, was without effect on the electrographic or clinical expression of the seizure. This is another indication of the overall integrity, including that in alternate pathways, of the seizure phenomena subserved by the sensorimotor system. c) Electrical stimulation 10/sec, 30V, 0.3 mA, of the ipsilateral ventral caudal globus pallidus caused reproducible, maximum expression of the electrographic and clinical phenomena of the focal seizure for the 90 second duration of the stimulus. This reflects the significance of this structure in the transmission of excitatory signals.

Projected Yield:

The long range future of this investigation has been entrusted to the Neurological Institute of Kyushu University, Fukuoka, Japan. It is being conducted by a cadre of scientists, specifically trained in the essential techniques while serving as members of the Laboratory of Experimental Neurology. To facilitate uninterrupted investigation, laboratory equipment that had been assembled here for this activity was made available to them through a loan from NINCDS, NIH, in January 1979. The emphasis has been shifted from focal motor seizures to seizures initiated in the temporal lobe with attention to the pattern of their spread to the hippocampus, amygdala, other parts of the limbic system

and the basal ganglia. The establishment of this new laboratory in Japan has the full administrative and scientific support of the Director of the Neurological Institute, Kyushu University.

Significance to Bio-Medical Research: Fresh insight into the pattern of subcortical propagation is afforded by this global appraisal of glucose utilization. Further, this established model of focal seizures will permit additional manipulation of cortical and subcortical activity by pharmacological or physical agents. This should provide new approaches to medical and/or surgical therapy for selected forms of the Epilepsies.

In addition this animal model of focal seizures should be of value in the interpretation of the use of deoxyglucose for positron emission scans in human epileptics.

Finally this activity represents a joint scientific effort between investigators of the United States and Japan.

2) DELAYED EFFECTS OF IONIZING IRRADIATION ON THE BRAIN OF THE MONKEY

Two models have been employed with focal and whole brain irradiation, respectively. Focal irradiation was used in studies of increased intracranial pressure and of the preferential spread of vasogenic edema. Whole brain irradiation was used primarily to simulate the effect of therapeutic doses on the normal brain. The principles that were demonstrated by the latter may be summarized as follows:

(1) The hallmark lesion is a minute focus of necrosis, that is widely scattered throughout the forebrain white matter. These lesions may appear as early as four or five months or as late as one to two years, following the radiation.

At any given time, after their appearance, they are seen to be in different phases of a cycle that begins with a punched out area, passes through phagocytosis, gliosis, and ends with mineralization. Individually, one may be in the initial phase while another is in the end phase. Larger at the outset, they are diminished in size as the cycle is completed.

In the aggregate, when there are a large number of the acute lesions, part of the effect is brain swelling from multiple minute breaks in the blood brain barrier. In the aggregate, when there are a large number in the stage of mineralization, the loss in brain substance is reflected by ventricular dilation.

(2) Accompanying, or perhaps preceding the discrete areas of necrosis are a variety of vascular abnormalities, the most notable of which are occasional absent or hyperplastic endothelial cells in adjacent capillaries. Quite apart from these are abnormal vascular channels making up patches of telangiectasia. These contribute to the brain damage and to the break in the blood brain barrier. The telangiectatic expression increases over time.

(3) Whether the minute necrotic lesions proceed to a predominantly healed phase, or increase in number with confluence that results in gross brain destruction, depends both on the initial exposure and the length of time after radiation. For example, the lesions from 1500 rads in a single dose, 6000 rads in a fractionated dose and 8000 rads in a fractionated dose, look very much alike at 26 weeks, but at 52 weeks those from the 6000 rads are all but quiescent while those from the other two exposures have resulted in widespread brain destruction.

(4) Malignant gliomas are a distinct rarity in the monkey. However, they have been found by others two or more years following whole body exposure to 600-800 rads with 55 Mev proton radiation. 1500 rads of supervoltage radiation in a single dose to the whole brain at puberty is beyond any therapeutic range, but the occurrence of neoplasms in this experimental group alerts one to the possibility of such a complication.

(5) The age of the host plays a significant part in the occurrence and the ultimate effect of the minute necrotic lesions. In the pubescent monkeys subjected to 6000 rads, fractionated dose, two out of four showed pronounced papilledema, prior to the 24th week. Subsequent optic atrophy was seen in one of these. A third showed blurred disc margins without measurable elevation in the nerve head. By contrast, in the adult monkeys subjected to 6000 rads, fractionated dose, only two out of the twelve that were killed at or beyond 24 weeks showed definite papilledema with a third showing blurred disc margins. As in the pubescent group, these funduspic changes were prior to 24 weeks.

The scattered minute necrotic lesions were seen in all four of the pubescent monkeys, killed at 26, 52, 68, and 78 weeks, respectively. By contrast the adult monkeys showed this phenomenon in only one out of three killed at 33 weeks, two out of three at 52 weeks and two out of three at 104 weeks. The third monkey in the last group showed eight lesions, all confined to the thalamus. Those at 33 and 52 weeks were predominantly in the intermediate phase; those at 104 weeks were predominantly in the late or mineralized phase. The increase in the telangiectatic expression over time was somewhat greater in the adult group than in the pubescent group.

(6) Within groups of the same age, with similar exposures, there are individual variations in susceptibility.

Significance to Bio-Medical Research and the Program of the Institute:
The principal advantage in simulating a therapeutic regime in a monkey model is to observe the effects in a brain uncomplicated by pre-existing pathology; e.g., that resulting from a neoplasm, surgical trauma or chemotherapy. This kind of information will be useful in the planning of therapeutic efforts in man, with attention to "risk-benefit" factors, and in the interpretation of fresh neurological findings following therapy. Further, there is a direct relevance to a major program of the Cancer Institute; i.e., the Brain Tumor Study Group, and we are privileged to share in their finding as this group is made aware of our results.

3) STRUCTURAL AND FUNCTIONAL SEQUELAE OF PENETRATING HEAD INJURY

The objectives are to provide orderly documentation of the Vietnam experience as regards the observation and management of head injuries incurred in combat; to uncover factors that have predictive value for cessation or persistence of initial neurological deficits; and to correlate the location and extent of brain loss, 12 years after injury, with the degree of impairment in sensorimotor, special sensory and cognitive function.

Registry: A registry for Head and Spinal Cord Injuries, as they occurred in military combat in Vietnam, was developed at the request of the Surgeon General of the U.S. Navy and was implemented with the cooperation of the Surgeons General of the U.S. Army and U.S. Air Force. The purpose was to insure uniformity of data collection and identify cases for present and future studies. The yield from field surgeons, 1967 to 1970, was 2,043 entries that included 1,683 head injuries, 329 spinal cord injuries and 31 combinations of the two. A rigorous appraisal demonstrated the uniformity and completeness of 1,540 head injury forms.

Records Review: After the injured returned to the United States, their intervening military and Veterans Administration hospital records were assembled by the Medical Follow-up Agency of the National Research Council and reviewed and coded by an experienced team of three neurological surgeons and three neurologists with the pertinent data being stored on magnetic tape by computer experts. Special attention was directed to the initial characteristics of the injury and the development and recession of sequelae. This activity began in July 1976 with the support of the Stroke and Trauma, and the the Intramural Research Programs, NINCDS. There are 1,221 cases on tape, and their analyses to date have yielded four published reports concerning posttraumatic epilepsy, cranioplasty, missile injuries crossing the midline, and language and motor deficits. Four reports are in preparation.

The cases under study are all men who were of uniform age (21.3 ± 3.2 years) at the time of injury. Their preinjury mental status as adjudged by the Armed Forces Qualification Test (AFQT) score included 5.2% in Category I, 28.2% in Category II, 40.2% in Category III, and 23.7% in Category IV. (Category I indicates the highest level of functioning). This distribution is comparable to that in U.S. Army military personnel in World War II.

Characteristics of the Injuries (n= 1,221)

Agent of Injury

Missile Fragments	940 (77.0%)
Gunshot Wounds	193 (15.8%)
Vehicular	49 (4.0%)
Other	39 (3.2%)

Severity of Injury

Alteration in Consciousness

Alert at examination	664 (54.4%)
Responded to Command	255 (20.9%)
Responded to Pain	303 (24.7%)

Depth of Injury

Fractures	210 (17.1%)
Single Lobe	510 (41.8%)
Multiple Lobes	501 (41.0%)

Comment: The material at hand includes 83% with local brain destruction as evidenced by penetration of the dura with cortical laceration. 45% were accompanied by immediate loss in consciousness. 25% were in coma, responding only to pain at time of examination, within six hours of injury. This makes possible the selection of cases by regions of focal damage, with and without functional evidence of diffuse brain damage. Such categories provide a background against which the natural history of early and late sequelae may be determined.

Selected Functional Sequelae Identified in the Records Review

Deficits	In Acute Phase	In Last Report
Motor	659 (54%)	383 (31.4%)
Visual	485 (39.7%)	393 (32.2%)
Dysphasia	316 (25.9%)	163 (13.3%)
Abnormal memory	187 (15.3%)	335 (27.4%)
Posttraumatic epilepsy	48 (3.9%) (1st wk)	396 (32.4%)

Comment: The yield from the records review was excellent in the details of the initial injury, its management, and the identification of sequelae. It was poor in uniform duration of follow-up: the records of 82% extended for 2 years, 45% for 4 years, and only 14% extended for 8 years. This variation in the time covered prevented an accurate appraisal of resolution of sequelae.

Protocol Development for In-hospital Examination: Under the auspices of the Veterans Medical Research Service and in conjunction with the

Walter Reed Army Medical Center (WRAMC), a plan is being developed in fiscal 1980 to bring 1,000 of these head injured subjects and 200 controls, matched for age, AFQT, and service in Vietnam, into a hospital setting for evaluation of their neurological status at this time.

The formulation of the hypotheses and the selection of the proposed examinations is being accomplished through a series of workshops that utilizes consultants in the disciplines of clinical neurology and neurosurgery, neuropsychology, speech pathology, computed tomography, anatomy, epidemiology and genetics. The refinement of the examinations will continue through the remainder of fiscal 1980.

Comment: The results of these examinations will yield three major blocks of information. The principal questions to be asked concern the relationship among these three sets of information. The first is the initial loss in brain substance, alteration in consciousness, and deficit in neurological function. The second is the focal and diffuse loss in brain substance, i.e., the anatomical sequelae, 12 years after injury, as evidenced by CT scan. The third is the functional sequelae, physical cognitive, behavioral, and seizure phenomena as determined by direct examination 12 years after injury. The management of these data and the analytic procedures to be used are being worked out with the Biostatistics Center, George Washington University.

Initial Agreements for Implementing the In-hospital Examinations: The Surgeons General of the Air Force, Army and Navy have agreed to provide the transport of the head injured veterans by Aeromedical Evacuation System; beds, lab and office at Walter Reed Army Medical Center; and computed tomography at the National Naval Medical Center, respectively. Operational funds are being sought from the Veterans Administration.

Significance to Bio-Medical Research and the Program of the Institute: The observations during the acute phase of injury in these cases are more uniform and probably more accurate than any previous series of comparable size. This provides an extraordinary opportunity for studies of prognostic factors and the natural history of disabilities in central nervous system trauma in man. The recent development of new techniques for evaluating functional deficits, e.g., those regarding language and memory and a mode for determining alterations in brain structure, i.e., the CT scan, will afford a unique opportunity for fresh insight into posttraumatic sequelae and into the function of the remaining brain.

There will be provided a thought provoking supplement to the acute head injury studies being supported by the Stroke and Trauma Program of NINCDS. Further, there will be identified subsets of the head injured group that should be of interest for subsequent studies within the Clinical Center Program, e.g., and estimated 100 cases with intractable seizures.

Publications in the Reporting Period

Wakisaka, S., O'Neill, R.R., Kemper, T.L., Verrelli, D.M., and Caveness, W.F.: Delayed Brain Damage in adult monkeys from radiation in the therapeutic range. Radiat. Res. 80: 277-291, 1979.

Meriowsky, A.M., Caveness, W.F., Rish, B.L., Dillon, J.D., Mohr, J.P., Kistler, J.P., and Weiss, G.H.: Definitive care of cerebral missile injuries crossing the midline. J. Milit. Med. Vol 145, No. 4: 246-250, 1980.

Kato, M., Malamut, B.L., Caveness, W.F., Hosokawa, S., Wakisaka, S., and O'Neill, R.R.: Local cerebral glucose utilization in newborn and pubescent monkeys during focal motor seizures. Ann. Neurol. Vol 7.: 204-212, 1980.

Caveness, W.F., Kato, M., Malamut, B.L., Hosokawa, S., Wakisaka, S., and O'Neill, R.R.: Propagation of focal motor seizures in the pubescent monkey. Ann. Neurol. 7: 213-221, 1980.

Hosokawa, S., Iguchi, T., Caveness, W.F., Kato, M., O'Neill, R.R., Wakisaka, S., and Malamut, B.L.: Effects of manipulation of the sensorimotor system of focal motor seizures in the monkey. Ann. Neurol. 7: 222-229, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02189-05 LEN																		
PERIOD COVERED October 1, 1979 through September 30, 1980																				
TITLE OF PROJECT (80 characters or less) Anatomical and Functional Sequelae of Penetrating Head Injury																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
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COOPERATING UNITS (if any) Walter Reed Army Medical Center National Naval Medical Center Research Service, Veterans Administration																				
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SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Objective:</u> To determine the loss in brain substance and the alteration in brain function 12 years after brain damage incurred during the Vietnam War. The resource for this is a Registry of 1500 Head Injuries compiled in the field by military surgeons from 1967 to 1970, and a subsequent Records Review carried out between 1975 and 1979. </p> <p> To achieve this objective a plan is being developed to complete this study by an in-hospital examination of 1000 of these subjects and 200 controls. The latter will utilize an unique approach to the loss or alteration in structure, i.e., computerized axial tomography, and other techniques, not available in previous studies of functional sequelae. </p>																				

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LAB/BRANCH <p style="text-align: center;">Laboratory of Experimental Neurology</p>														
SECTION 														
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SUMMARY OF WORK (200 words or less - underline keywords) <p>Twenty-one adult <i>Macaca mulatta</i> that received 6000 rads of supervoltage X-irradiation in 30 fractions, alternate sides of the brain being exposed on alternate days, showed widely scattered minute necrotic lesions in the forebrain white matter in one out of three monkeys at 33 weeks, two out of three at 52 weeks, and two out of three at 104 weeks. With passage of time there was an increasing number of lesions with a trend toward mineralization except for those that extended into the cerebral cortex. Accompanying the necrotic lesions were both focal vascular endothelial hyperplasia and patches of telangiectasis.</p> <p style="text-align: center;">This project has been completed and the results published.</p>														

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TITLE OF PROJECT (80 characters or less) <div style="text-align: center;">Thermal Manipulation of Paroxysmal Neuronal Activity</div>																		
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SUMMARY OF WORK (200 words or less - underline keywords) <p style="text-align: justify;"> In the study of subcortical structures that are brought into play with the propagation of paroxysmal activity from a penicillin focus in the motor cortex of the monkey, a question of importance is: In what way are neuronal aggregates essential to the full development of the <u>experimental focal seizure</u>? In seeking an answer, neuronal blockade of structures with previously demonstrated involvement was brought about by a stereotactically controlled <u>cryoprobe</u>. The instrument at hand permits graded cooling at its tip that is 0.1 cm in diameter and monitored by a microthermocouple. By using destructive degrees of cooling in selected subcortical areas, principally the ipsilateral globus pallidus, the progression of the paroxysmal activity was not affected. </p> <p style="text-align: center;">The results from the above have been published and the project completed.</p>																		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02428-01 LEN												
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TITLE OF PROJECT (80 characters or less) Anatomical Corollary for Computed Tomography														
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SUMMARY OF WORK (200 words or less - underline keywords) <p>The objective is to provide templates of anatomical structures from the human head at intervals of 1 mm from base to vertex. The horizontal sections will be parallel with a plane passing through the glabella and external auditory meatus. These will be used in interpreting the anatomy represented in CT scans obtained in the same horizontal plane, at 5 mm intervals, from base to vertex.</p> <p>To bring this about an LKB 2250 PMV Cryo-microtome is being modified to accommodate the human head. This instrument will be installed in the Uniformed Services University of Health Sciences where the study will be conducted in conjunction with the Department of Anatomy. The product of this effort is primarily intended for the interpretation of CT scans. A secondary objective is to provide a definitive Atlas for use by others.</p>														

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neurochemistry
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980
Laboratory of Neurochemistry, Intramural Research
National Institute of Neurological and Communicative
Disorders and Stroke
Janet V. Passonneau, Chief

The Laboratory of Neurochemistry is composed of four sections, the Section on Cellular Neurochemistry, the Section on Neurochemical Pharmacology, the Section on Enzymes, and the Section on Neuronal Development and Regeneration. These sections are engaged in a variety of projects.

Section on Cellular Neurochemistry

The Section on Cellular Neurochemistry has three projects currently in progress:

a. Metabolic Profiles in Normal and Diseased Retina.

A study is in progress of the metabolite distribution in the retinas of dark-adapted frogs, and after 2 minutes or 2 hours of exposure to light. The layered structure of the retina makes it a suitable choice for the study of metabolite changes in the different cell populations, and even parts of the same cells; for example the outer segments, inner segments and nuclear layers of the photoreceptor cell. The exposure of the photoreceptors to light results in hyperpolarization; consequently the effect on the photoreceptor cells would reflect a quiescent rather than active state. The second order neurons in the retina have a mixed response, both hyper- and depolarization. The changes in metabolites such as ATP and P-creatine reflect the activity of the retinal components: the photoreceptors show an increase in energy reserves and in the inner retina there is no change.

Future plans include the use of drugs to destroy selectively portions of the retina (streptozoin destroys the rod outer segments) and retinopathies such as those which occur in diabetes, gyrate atrophy and ischemic injury.

b. Coordinate Effects of Amphetamine on Brain Energy Metabolism and Protein Synthesis.

Amphetamine has been shown to have potent glycogenolytic action in the brains of rodents. High-energy phosphate reserves are also affected. Both of these changes have been associated with an increased cerebral metabolic rate.

Amphetamine also causes a transient inhibition of protein synthesis, related to the ambient temperature. The drug produces hyperthermia in mice kept in a warm environment and hypothermia in mice kept in a cold environment.

There is evidence that the inhibition of brain protein synthesis by amphetamine is dependent on the hyperthermic response of the drug. However, hypothermia induced by external heating of the animal does not in itself cause a reduction in protein synthesis. It is presumed that other effects of amphetamine administration interact with the concomitant hyperthermia to inhibit protein synthesis.

The relationship of the metabolic effects of the drug to body temperature responses must also be considered. There is evidence that adequate energy supply in the brain may be related to the regulation of protein synthesis. If there is a temperature-dependent component of amphetamine effects on brain energy metabolism, these metabolic consequences could be related to the effect on brain protein synthesis.

Other experimental treatments which alter brain metabolism also alter protein synthesis (electroconvulsive shock, convulsant drugs, spreading depression). A common metabolic response may be responsible for the reduction in protein synthesis.

The plans include a survey of amphetamine effects on mice. Two strains have already been identified, one of which is sensitive, and the other relatively insensitive to amphetamine administration in terms of changes in body temperature.

The energy reserves of brain, the adenine and guanine nucleotides, including the cyclic nucleotides will be examined with regard to the response to amphetamine. The changes in protein synthesis will be also characterized to see whether a relationship exists with the metabolic effects. Polysome profiles and amino acid incorporation into brain will be investigated.

c. Characterization of Anaerobic Metabolism of Normal and Neoplastic Astrocytes in vitro.

Several cell line of astrocytes, normal or transformed, are being investigated concerning their metabolic characteristics. These include: rate of anaerobic metabolism, metabolite profiles, and regulatory mechanisms. Of special interest is the formation and utilization of glycogen. Primary astrocytes prepared from rat brain, and several transformed lines, either spontaneously or chemically or virally induced, are being investigated.

The rates of glucose uptake, and lactate and pyruvate efflux have been measured to compare the utilization of glucose in normal and transformed cells. The accumulation of glycogen and its degradation in response to glucose deprivation, or to agents that stimulate cyclic AMP formation, and thus the glycogenolytic cascade, are being investigated. The glycogen synthetic and degradative enzymes both exist in active and inactive forms. The cell lines differ at least qualitatively in their activity and conversion of these enzymes. In some lines, glycogen synthase, the synthetic enzyme appears to be in the inactive form. These cells are apparently deficient in the enzyme glycogen synthase phosphatase, which converts the inactive phosphoenzyme to the active dephosphoenzyme. Other lines appear to have a deficiency in the phosphorylase

phosphatase which converts the active phosphoenzyme to the inactive dephosphoenzyme.

The concentration of glycogen, and the activities of the enzymes and the relative amounts of the active and inactive forms of enzymes are being investigated in several cell lines. The objective is to find clones which are altered in every genetic locus involved in glycogen anabolism and catabolism.

Section on Neurochemical Pharmacology

a. Cerebral Ischemia.

Previous studies using the Gerbil model of ischemia have provided evidence on potential pathogenic sites that could lead to irreversible brain damage. The emphasis of the research now centers on these biochemical aberrations. It has been established that cyclic AMP increases and cyclic GMP decreases during an ischemic episode, but that both cyclic nucleotides increase significantly during the early stages of recirculation. In order to evaluate the molecular mechanisms involved in these changes, rat brain slices are being incubated in a medium free of both glucose and oxygen to mimic the ischemic condition and in a medium with oxygen and glucose for recovery. The cyclic nucleotide response in vivo can be duplicated in vitro and attempts to identify the stimulus to the changes in the cyclic nucleotides have to date been essentially negative, suggesting that it may be more complex than anticipated.

Other biochemical perturbations that are currently being examined are the overshoot of glycogen and P-creatine and the restitution of the adenylate pool during the recovery period. These are being monitored both during and after intervals of ischemia that are consistent with recovery and those that are not. By using in situ fixation, the regional response can be evaluated and eventually the phenomenon of selective vulnerability as manifested by the H-1 and H-3 neurons of the hippocampus will be investigated.

b. Experimental Seizures.

The changes in energy metabolism and cyclic nucleotides during electrically and chemically-induced seizures have been demonstrated in regions, in discrete layers within a region and in single cells. The preservation of ATP in Purkinje cells of the cerebellum but not in pyramidal tract neurons of the cerebral cortex following maximal electroshock reinforces the importance of energy metabolism in the expression of seizures. Both cyclic nucleotides increase during the excitable stages of convulsions; but however cyclic AMP is thought to be a consequence of the seizure, and there is evidence to suggest that cyclic GMP may play a role in the events that lead up to and trigger a seizure. The significance of the aforementioned metabolite changes to the onset, propagation and termination of a seizure is being examined using a kindling model. Whereas previous studies have relied on behavioral changes as an indicator of seizure activity, the electrical activity in the kindled animal will be monitored and a more precise determination of the relationship between metabolite changes and neuronal activity can be made.

Generally, anticonvulsants depress the levels of cyclic GMP in the cerebellum and have little effect on the levels in the cerebral cortex. However valproate has been shown to consistently increase the cyclic GMP in the cortex by 50%. Since cyclic GMP in the cerebral cortex is elevated during hyperexcitable states, the effect of valproate may be a side effect unrelated to its anticonvulsive properties. Another CNS depressant which increases the concentrations of cortical cyclic GMP is halothane. The levels of cyclic GMP are restored to near those of control even though the animals are still anesthetized. So like valproate, the elevation of cyclic GMP appears to be dissociated from the principle action of the drug. The role of cyclic GMP in the cortex may explain, in part, some of the untoward effects of these agents.

c. Hibernation.

The adaptation of the hamster to extremes in the environment has been useful in investigations on 1) fixation artifacts that arise in brain and 2) the mechanism whereby the brain can reversibly switch to an electrically quiescent state. The changes in the brain metabolites from hibernating hamsters are as follows: GABA and lactate are elevated and cyclic GMP is depleted. The energy status of the brain as reflected by the high-energy phosphates is as high if not higher than the normothermic brain; however, these differences may be attributed in part to fixation artefact. The levels of cyclic AMP, glycogen and ATP were not affected to any great extent. Thus, the hibernating hamster is a good model for evaluating cerebral metabolism in a naturally occurring dormant stage.

Section on Neuronal Development and Regeneration

The Section on Neuronal Development and Regeneration is studying the use of nervous tissue allografts (i.e., a graft between genetically different members of the same species) to aid in the repair of injured nerve tissue. Previously it was found that a nerve allograft fails because the transplantation antigens it bears evokes an immune reaction by the host which culminates in the destruction of the allograft. Further work has shown that the immune response to an allograft can be overcome by making the host immunologically tolerant to the antigens of the tissue donor. As a consequence of tolerance-induction, it was possible to bridge a 4 cm gap in the injured peroneal nerve of host rats with an allograft. In that study the axons of tolerant rats regenerated through the allograft and reinnervated the muscles that were denervated as a result of the nerve injury to the host. Tolerance and successful nerve repair was attained regardless of whether the nerve allograft contained minor or the more potent major antigens. Since allogenic Schwann cells would be expected to survive and function (i.e., myelinate axons) in tolerant rats, it was of interest to abolish tolerance and determine the fate of host axons after the allogenic cells surrounding them were rejected. When this was done it was observed that host axons in the allograft degenerated and that a second wave of host axonal growth was occurring. A study is underway to determine the outcome of this second axonal regenerative attempt. It would be of interest also to devise ways to prevent rejection episodes which might occur during states which promote tolerance. A study is in progress to evaluate clinically

used immunosuppressive drugs. Cyclosporin-A was found the most effective agent in that it could prevent the rejection of neurons and Schwann cells in nerve and myofibers in muscle allografts. Cyclosporin-A appeared to be non-toxic to the treated rats and host axons could regenerate through a nerve allograft. The study with Cyclosporin was not long enough to permit regeneration to denervated tissue since initially the goal was to determine the dose of Cyclosporin-A that was immunosuppressive. A long-term study with Cyclosporin-A is planned which will include cessation of the drug later on. It might be that some form of tolerance can be induced by Cyclosporin-A.

A related series of experiments is being performed to attempt to alter the transplantation antigens present on an allograft. This might be achieved by treating the donor or donor tissue (e.g., in culture) prior to insertion of the allograft into the host. Leukocytes in an allograft are a potent immunogenic stimulus and their removal by leukotoxic drugs or radiation could be beneficial. Antigens might also be masked by treatment with lectins which could then alter the response to an allograft.

A second project of this section is concerned with trophic neuron function. In several instances, the neuron is responsible for causing the development or maintaining the integrity of target tissue. This effect is believed to be mediated by a factor(s) released at the nerve ending. The ultimate goal is to determine the nature, specificity and plasticity of trophic neuron function. The main effect will be devoted to an attempt to see if a nerve extract can cause taste bud development in culture. An "in situ" study will investigate whether the non-taste nerve fibers of motor neurons can penetrate into oral epithelium and induce taste buds. It could be that the basement membrane of oral tissue regulates what type of nerve enters it. Finally, grafts of neurons (i.e., in ganglia) and human tongue will be cotransplanted into immunosuppressed animals (or culture) to determine if cross-species formed buds will develop. Rat and mouse cross-species buds already have been produced. Ultimately an attempt will be made to see why patients with the disease familial dysautonomia fail to develop taste buds or fungiform taste papillae.

Section on Enzyme Chemistry

a. Transient kinetic studies of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

³²P-ATP and its dephosphorylation are measured by means of a rapid quenching apparatus with time resolution of about 3 msec. This has been applied to a study of the mechanism of vanadate inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. Vanadate has been shown to form a complex with the enzyme which cannot be phosphorylated. Vanadate is considered by some workers as a possible physiological regulator of sodium transport.

b. A method has been developed to permit quantitative endgroup analysis of insoluble membrane proteins. This method will be applied to the problem of determining the subunit stoichiometry of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

c. A method has been developed for preparing the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from

different tissues under standard conditions. This will be applied to resolve the issue of reported differences in ligand-binding stoichiometries among (Na + K)-ATPase preparations from different sources.

d. Preliminary experiments have been conducted on the preparation and assay of concentrates of low molecular weight fractions of tissue extracts from brain and electric organ. We plan to follow up on several reports of the presence of endogenous regulators of (Na⁺ + K⁺)-ATPase activity in brain.

e. We are employing an analog of ATP, ATP- γ -S, to study the relation between phosphorylation and conformational transitions which constitute the ion transport process between the phosphorylation of the enzyme and the subsequent conformational transitions that constitute the transport process. We have shown that this analog has a high affinity for the ATP-binding site and transfers its terminal thiophosphoryl group to the enzyme in a Na⁺-dependent reaction. Preliminary indications are that the conformational equilibria of the thiophosphorylated enzyme is different than that of the normally phosphorylated (Na⁺ + K⁺)-ATPase. These observations will be pursued by systematic studies of the steady-state kinetics of these reactions.

f. Studies have been initiated to investigate certain aspects of calcium metabolism in electric organ tissue. Studies of the binding of the calcium regulatory protein, calmodulin, to electric organ membranes have been carried out. In addition, a Ca⁺⁺-activated membrane ATPase has been identified in these membranes. Both of these observations will be the subject of further study.

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SUMMARY OF WORK (200 words or less - underline keywords) <p> This project is an investigation into the mechanism and structure of the $(Na^+ + K^+)$-ATPase. Studies are proceeding along the following lines: (a) measurements of the <u>transient kinetics</u> of the phosphorylation and dephosphorylation of the $(Na^+ + K^+)$-ATPase; (b) experiments designed to determine the <u>subunit structure</u> and stoichiometry of the functional unit of the $(Na^+ + K^+)$-ATPase; (c) studies to determine the <u>stoichiometries</u> of ligand-binding in relation to the subunit structure of the $(Na^+ + K^+)$-ATPase; (d) investigations into the <u>possible existence</u> of endogenous regulatory components acting upon the $(Na^+ + K^+)$-ATPase in brain and other tissues; (e) studies of the relation of <u>enzyme phosphorylation</u> to the conformational transitions of the enzyme cycle; (f) studies of the kinetic properties of the ATP hydrolysis catalysed by the $(Na^+ + K^+)$-ATPase in the absence of K^+. </p>																										

Project Description:

Objective: These studies are all designed to obtain a detailed description of the structure of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and of the molecular events that produce sodium ion transport.

Methods:

The various subprojects approach the general objectives via different strategies and methods: a) The transient kinetic studies employ a specially designed rapid-quenching device for denaturing and isolating samples of radioactively-labelled enzyme after millisecond reaction times. Amounts of phosphoryl-enzyme and of inorganic phosphate present as a function of time are determined simultaneously. Reaction constants are determined by computer fitting of data to differential equation models. Various reagents are used to investigate effects of enzyme modifications on the reaction kinetics. The apparatus permits two-stage addition of reagents so that, e.g. the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ may be phosphorylated in the absence of K^+ and K^+ may be added in the second stage mixer which then permits determination of the time course of dephosphorylation.

b) Methods are under development with the object of obtaining a more precise determination of the stoichiometry of the peptide chains that constitute the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. These methods involve purification of the enzyme, precise determination of amino acid composition and quantitative determination of N-terminal amino acids. Measurements will be made on both intact, functional enzyme and isolated peptide chains. This data will be complemented by measurements of molecular weight of the functionally-intact $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ by physical methods. Methods under consideration include light-scattering, osmometry, and radiation target inactivation.

c) Ligand-binding studies employ an equilibrium technique developed in this laboratory which utilizes the miniature ultracentrifuge. In addition various conventional filtration methods are used in the measurement of the relation of substrate, activator, and inhibitor binding sites to the overall structure of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

d) In conjunction with the ligand-binding studies, we are examining the possibility that endogenous tissue components may exist that can modify the various ligand-binding properties of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. These studies employ the techniques outlined in (c) above.

e) The energy transferred to the enzyme consequent to its phosphorylation by ATP is utilized in the ion-pumping process. This is accompanied by conformational transitions of the enzyme protein structure. We are employing structural analogs of ATP which have different chemical reactivities with respect to their abilities to bind to the enzyme and/or to react subsequent to binding. Some of these differences are reflected in the conformationally-determined properties of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. The

various partial reactions catalysed by the enzyme are used to monitor these differences. It seems probable that valuable insights into the mechanism by which conformational transitions are coupled to the enzyme reaction can be attained by this strategy.

Major Findings:

- a). Transient kinetic studies (Hobbs, Froehlich, Albers). We have investigated the mechanism of vanadate inhibition of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. Vanadate is normally present in tissues in concentrations that are detectable inhibitory to the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. These experiments demonstrate that vanadate is a potent inhibitor of the Na^+ -dependent enzyme phosphorylation. The primary effect was found to be on the extent rather than the rate of the phosphorylation reaction. K^+ is necessary for the vanadate inhibition. The non-competitive characteristic indicates that vanadate forms a complex with the enzyme that cannot be phosphorylated by ATP.
- b). Subunit structure (Chock, Albers). Recent efforts on this phase of the project have been devoted to developing methods for measuring the ratios of N-terminal amino acids to total amino acids in the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and other membrane proteins. This will permit accurate determination of the molecular weights of large polypeptide chains that constitute the enzyme subunits. In addition it will allow determination of the stoichiometry of the subunits in the functional enzyme. These methods are based upon a modified Edman reagent forming intensely colored amino acid derivatives that are identified and quantitated by high performance liquid chromatography. A remaining technical problem arises from the insolubility of membrane proteins. We are currently investigating techniques for preparing thin films of protein over a solid matrix. This approach seems promising for efficient reaction of protein with reagent and extraction of derivatives.
- c). Ligand-binding studies (Krishnan, Albers). Recent work on this phase of the project has concentrated on development of a standard method for $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ preparation that will be suitable for a wide range of enzyme sources. Differences in the preparative methods have been a source of uncertainty in the interpretation of much of the available data on ligand binding to the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. We have studied the effects of deoxycholate and other detergents on preparations from brain, kidney, electric organ, etc. and developed a standardized preparative method. The major technical problem is the presence of vesicles in membrane preparations which have variable stabilities depending upon the source. Vesicles must be rendered permeable to all ligands before valid binding experiments can be performed.
- d). Endogenous regulatory factors (Krishnan). Peptides from several sources have been screened as possible competitors or modulators of radio-active ouabain binding to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. Results to date have been negative or equivocal. A difficulty in assaying peptides is to insure that they are free of ammonia or potassium ions, either of which can depress ouabain binding. Some preliminary work has been done on the preparation of brain and electric organ concentrates of low molecular weight material in salt-free

condition, prior to assay.

e). Enzyme phosphorylation related to conformational transitions (Krishnan, Albers). Our initial observations that led to this study was that the compound, ATP- γ -S, substitutes effectively for ATP in catalysing the binding of ouabain to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. We have since shown that ATP- γ -S has a high affinity for the enzyme, can transfer the terminal thiophosphoryl group to the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in a Na-dependent reaction, but is only slowly hydrolysed by the enzyme. We have evidence that the thiophosphorylated enzyme remains largely in the "E₁" state in contrast to the phosphorylated enzyme. However the maximum level of thiophosphorylation is lower than the level of phosphoryl-enzyme that may be obtained under the usual assay conditions.

Proposed course:

a). Transient kinetics. Our previous studies have established that the antibiotic, oligomycin, stabilizes the "high-energy" form of the phosphoryl enzyme (E₁-P). Preliminary transient kinetic experiments have confirmed this data. We have shown additionally that oligomycin only binds to the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in the presence of sodium ions. We plan to extend these experiments and to carry out similar experiments with other agents which alter the conformational equilibria of the enzyme. These experiments should complement the studies utilizing ATP- γ -S in increasing our understanding of the manner in which metabolic energy is coupled to ion transport.

b). Subunit structure. We expect to apply the strategy of end-group analysis to determine the peptide chain stoichiometry, to investigate the possible relationship of the proteolipid to the functional enzyme and to assess the relative purity of different enzyme preparations. We also plan to initiate studies aimed at more precise determination of the molecular weight of the functional unit of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. This will require a physical method: membrane osmometry, light scattering, low-angle x-ray diffraction and target inactivation analysis are possible approaches to this problem.

c). The ligand-binding study are to be correlated with the subunit studies. We wish to determine whether the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ can function as a monomeric structure or whether oligomeric interactions are an intrinsic part of the mechanism. The number of nucleotide-binding sites, phosphorylation sites and other modifier sites will be reexamined as more precise structural data becomes available. For example, it has been claimed that there is only one high-affinity nucleotide-binding site per two catalytic subunits. However this conclusion relies on measurements of molecular weight and subunit concentrations that are prone to large errors. Similarly there are reported species differences in the ratio of ouabain-binding to phosphorylation sites which have not yet found a structural explanation.

d). Endogenous regulatory factors. We shall continue to test for the existence of factors of biological origin that may modulate $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

activity or binding properties. We intend to pursue some systematic testing of low molecular weight fractions from brain and electric organ tissues.

e). Relation of enzyme phosphorylation to transitional conformations. The ability of ATP- γ -S to thiophosphorylate the enzyme provides a unique and unexploited tool for investigating the mechanism by which the transport process is driven by the phosphorylation reaction. The chemistry of acylthiophosphates has not been studied as it applies to biological system. The thiophosphate analog of ATP has a lower pK than ATP. However the free energies of and hydrolysis has not been determined. One of the first objectives will be to establish the extent of parallelism between the thiophosphorylated and the phosphorylated enzyme. This will entail characterization of the thiophosphorylated enzyme in terms of its pH stability and the nature of its peptic "fingerprint", both of which have previously been determined for the phosphorylated enzyme. Steady-state kinetic experiments and examination of the ability of ATP- γ -S to replace ATP in the various "partial reactions" of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ will also be determined. Depending upon the outcome of these experiments, work may be extended to an examination of the transient kinetics of the thiophosphorylation reaction. One tentative hypothesis is that the thiophosphorylated enzyme converts from E_1 - to E_2 -forms much more slowly than does the phosphorylated enzyme. This could be most directly examined by transient kinetics.

Significance:

The $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ is the enzymatic machinery of active sodium ion transport. This process generates the cellular membrane electrical potential and maintains the principal cellular ionic gradients. These ionic gradients are the basis of the nerve action potential, of neurotransmitter uptake, and of numerous other Na^+ -dependent transport functions. The sum of these functions constitute the major metabolic work of brain and nerve tissues.

A detailed knowledge of the molecular events of the Na^+ active transport system is fundamental to understanding brain biochemical function. Little is as yet known about the regulatory mechanisms of Na^+ -transport. These must be matched to the many different processes that are known to be Na^+ -dependent, and must be closely related to the regulation of brain energy production.

The structural and binding studies outlined here should contribute to an explanation of the functional differences among the $(\text{Na}^+ + \text{K}^+)\text{-ATPases}$ of different tissues and the physiological regulation of sodium transport.

Publications:

Hobbs, A.S., Froehlich, J.P. and Albers, R.W.: Inhibition by vanadate of the reactions catalysed by the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$: a transient kinetic characterization. J. Biol. Chem. in press (1980).

Hobbs, A.S. and Albers, R.W.: The structure of proteins involved in active membrane transport. Annu. Rev. Biophys. Bioeng. 9: 259-291, 1980.

Hobbs, A.S., Froehlich, J.P. and Albers, R.W.: Potassium-induced changes in phosphorylation and dephosphorylation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. J. Biol. Chem. 252: 3395-3402, 1980.

Krishnan, N. and Albers, R.W.: Modification of the rate of ouabain binding to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ by lithium ions. J. Neurochem. in press (1980).

Lopez-Cardozo, M. and Albers, R.W.: Relationship between the 4-aminobutyrate bypath and the oxidation of 2-oxoglutarate in rat brain mitochondria. J. Neurochem. 33: 1259-1265, 1979.

Rodichok, L.D. and Albers, R.W.: The effect of γ -aminobutyric acid on substrate-level phosphorylation in brain mitochondria. J. Neurochem. 34: 808-812, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01586-13-LNC
PERIOD COVERED <p style="text-align: center;">October 1, 1979 to September 30, 1980</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Trophic Function of Neurons in the Peripheral Nervous System</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; margin-top: 20px;"> PI: A. A. Zalewski LNC, NINCDS </div>		
COOPERATING UNITS (if any) <p style="text-align: center;">T. H. Oh, Department of Anatomy, University of Maryland</p>		
LAB/BRANCH <p style="text-align: center;">Laboratory of Neurochemistry</p>		
SECTION <p style="text-align: center;">Neuronal Development and Regeneration</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">0.3</p>	PROFESSIONAL: <p style="text-align: center;">0.2</p>	OTHER: <p style="text-align: center;">0.1</p>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINDRS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p> The purpose of this project is to determine the specificity and plasticity by which neurons regulate the development, maintenance or regeneration of target tissue. In one study, neurons in <u>grafts of sensory ganglia</u> were isolated in the anterior chamber of the eye for one year at which time tongue grafts were added. The isolated neurons responded to the tongue tissue by growing axons into it where they induced <u>taste bud formation</u>. In another study, it was shown that the rejection of neurons in rat sensory ganglia could be prevented by treatment with the <u>immunosuppressive agent Cyclosporin-A</u> and that these surviving neurons could <u>regenerate axons</u> into tongue tissue and induce the formation of taste buds. Cyclosporin-A was also found capable of preventing the rejection of <u>muscle allografts</u> which ultimately became reinnervated by host axons. Muscle in a rat with congenital absence of the peroneal nerve was reinnervated by another nerve to see if muscle spindles (stretch receptors) would appear. No spindles were found indicating that if innervation is missing during a critical period of embryonic muscle development spindle receptors do not form. </p>		

Project Description:

Objective: Trophic nerve function refers to the ability of a neuron to induce and/or maintain the development of an end-organ. It is currently believed that the neuron synthesizes a factor(s) that is transported via the axoplasm to its nerve terminal where it is released and regulates end-organ integrity. This function of nerve readily explains why tissues such as taste buds (which differentiate from ordinary lingual epithelial cells) and skeletal muscle fibers degenerate and disappear after elimination of their innervating nerve fibers and why these tissues regain their health after nerve regeneration. The purpose of this project is to elucidate the nature, specificity and plasticity of trophic nerve function. Sensory neurons in grafts of ganglia were isolated in the anterior chamber of the eyes of rats for one year to determine if isolated neurons would maintain their ability to respond to target tissue (i.e., addition of a tongue graft to the eye). The immunosuppressive drug Cyclosporin-A (CsA) was administered to rats to see if it could prevent the rejection of neurons in allografts (graft between genetically different members of the same species) of ganglia. In another study, CsA was tested to find out whether it could prevent the rejection of allografts of skeletal muscle. A study was also performed to investigate whether muscle spindles (i.e., sensory stretch receptors of skeletal muscle) could develop in muscle congenitally lacking innervation. Spindle formation requires innervation and the point of this study was to distinguish whether spindles could still differentiate postnatally after ingrowth of nerve or whether a period of embryonic specificity existed which if passed precluded spindle formation regardless of subsequent innervation.

Methods Employed:

A) Taste Bud Study: Inbred Brown Norway rats were used and sensory vagal nodose ganglia transplanted into the anterior chamber of the eyes of host rats. One year later a graft of Brown Norway tongue tissue was added to eyes containing ganglia (tongue grafts were placed over the peripheral end of ganglia). Tongue grafts were examined 35 days later for the presence of nerves and taste buds.

Allografts of sensory ganglia from Brown Norway rats were transplanted into the eyes of Fischer rats that were given the immunosuppressive drug Cyclosporin-A (25 mg/kg, subcutaneous) or that went untreated. Isografts (non-antigenic grafts because they are taken from the same inbred strain as the host rat) of tongue of Fischer rats were added and 30 days later the ganglia examined for surviving neurons and the tongue tissue for taste buds.

B) Muscle Study: Cyclosporin-treated or untreated rats received an allograft of the extensor digitorum longus (EDL) muscle from Brown Norway rats that was placed into the site formerly occupied by the excised EDL of the Fischer host. Thirty days later all muscle allografts were examined for the presence of muscle fibers.

A normal branch of the tibial nerve was implanted into the tibialis anterior (TA) muscle of a group of 10-day old rats that congenitally lack the peroneal nerve which normally innervates the TA muscle. Three months later the TA was checked for innervation of the muscle fibers (extrafusal fibers) as well as for the development of muscle spindles (intrafusal fibers). It bears mentioning that spindles were absent in denervated but present in innervated 10-day old TA muscle.

Major Findings:

A) Taste Bud Study: Neurons survived in one-year grafts of sensory ganglia and then, upon exposure to their target tissue, regenerated axons into it and induced taste bud formation. Cyclosporin-A treatment prevented the immunological rejection of neurons in allografts whereas in untreated rats all neurons were absent. Moreover, surviving allogenic neurons could extend axons into tongue tissue and cause taste bud development. It was also noted that allogenic Schwann cells survived after Cyclosporin-A treatment and that these cells myelinated axons.

B) Muscle Study: Muscle allografts survived and regenerated myofibers in Cyclosporin-A treated rats. In addition, some neuromuscular connections were already being formed by host axons and surviving allogenic myofibers. No muscle fibers survived in allografts in normal, untreated rats. Implantation of a normal nerve into congenitally uninnervated muscle failed to induce muscle spindle formation although muscle fibers did become innervated.

Significance:

A) Taste Bud Study: The ability of one-year isolated sensory neurons to respond to target tissue by reinitiating regeneration implies that neurons do not lose their ability to regenerate and that target tissue may elaborate a factor(s) which stimulates axonal growth. The survival of neurons in allografts after Cyclosporin-A treatment shows this drug is effective in preventing neuronal rejection. Cyclosporin-A may be useful if neurons in brain tissue allografts prove clinically useful and require immunosuppression.

B) Muscle Study: Cyclosporin-A prevented the rejection of muscle allografts which subsequently began to become reinnervated by host axons. It may now be possible to use muscle allografts to replace traumatized, diseased or congenitally absent muscle. The failure of muscle spindles to develop postnatally after ingrowth of sensory and motor axons demonstrates that there is a critical period of muscle development where spindle induction occurs and which can not be reduplicated.

Proposed Course of Project:

A) Taste Bud Study:

1) An effort will be made to grow taste buds in culture. The prime study will be to determine whether a nerve extract

rather than the intact nerve can induce buds.

2) Denervated tongue tissue will be reinnervated in situ by motor nerve fibers to determine if these fibers actually enter the tongue epithelium. This fact has yet to be reported and it might be that basement membrane prevents non-sensory nerve fibers from entering lingual epithelia.

3) A study will be performed to ascertain the role of epithelium and connective tissue in forming taste buds. Tongue and skin will be trypsinized to separate epithelium and dermis and recombinations such as skin epithelium and tongue dermis will be made to determine if this permits bud regeneration.

4) Experiments will be performed to determine whether human taste buds can be formed in grafts in the anterior chamber of the eye. Human buds might be grown in hosts (rats) that are immunosuppressed with antilymphocyte serum (ALS). Buds usually regenerate within 5 weeks and this period might be short enough to avoid encountering toxic effects of ALS. This study, if successful, would be repeated on human tongue tissue obtained from patients with disease familial dysautonomia who congenitally lack taste buds.

B) Muscle Study:

1) The spinal cord region and sensory ganglia which make up the peroneal nerve will be studied in rats with the congenital absence of this nerve. An attempt will be made to determine if the neurons making up this nerve are present or absent. A quantitative count of sensory ganglia should prove informative while localization of peroneal motor neurons in the cord probably can best be done by retrograde tracer studies (i.e., horseradish peroxidase etc.). Since most rats have a unilateral absence of the nerve it would be possible to apply horseradish peroxidase to the cut end of the normal nerve and, after retrograde transport of the enzyme, identify the location of peroneal motor neurons in the spinal cord. These labeled, normal peroneal motor neurons would then serve as the standard with which to compare the presence, number and distribution of motor neurons on the contralateral side of the spinal cord in which the peroneal nerve is absent.

Publications:

(1) Oh, T. H., G. J. Markelonis, P. J. Rier, and A. A. Zalewski. 1980. Persistence in degenerating sciatic nerve of substances having a trophic influence upon cultured muscle. Exp. Neurol. 67: 646-654, 1980.

(2) Zalewski, A. A. Survival, regeneration, and trophic function of neurons in 1-year transplants of sensory ganglia. Exp. Neurol. 68: 390-394, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02006-08 LNC
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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of Metabolism in Glioma and Neuroblastoma Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.V. Passonneau Head, Sect. on Cellular Neurochem. LNC NINCDS
OTHER: C.J. Cummins Staff Fellow LNC NINCDS
W.D. Lust Head, Sect. on Neurochem. Pharm. LNC NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Neurochemistry

SECTION

Section on Cellular Neurochemistry

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.4	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are being carried out on primary cultures of astrocytes and on several transformed glial cell lines in vitro. Primary astrocytes are derived from rodents either by viral transformation of primary cultures (HSV-RPA cell lines, S22) or from tumors derived from human pathological specimens, (Mills, Green), or from tumors induced in rodents in situ and subsequently established in vitro, (C6, B9, B82). The levels of key metabolites, glucose, glycogen lactate, pyruvate, ATP, P-creatine, GABA, glutamate, and cyclic nucleotides were measured in cells and medium after refeeding confluent cells with MEM + glucose. The regulatory enzymes of glycogen metabolism, synthase and phosphorylase have been measured, and the various cell lines have been characterized according to the presence or absence of glycogen and the regulatory enzymes of glycogen metabolism. Primary cultures and HSV-RPA, shown enzyme activities comparable to brain, and a high level of glycogen after addition of glucose. Mills and Green's cell lines shown an unregulated high level of glycogen; and other cell lines, B9, B82, ES22 synthesize relatively low levels of glycogen. These findings are hypothesized to result from the absence of one or both of the regulatory enzymes metabolism.

Project Description:

Objectives: To investigate the regulation of glycogen metabolism of normal and transformed astrocyte cell lines, to collect cell lines altered in glycogen metabolism, and to construct cell lines by mutagenesis and cloning, which are altered at specific loci of glycogen metabolism.

Methods Employed: The cells are grown in plastic dishes using modified Eagle's Medium containing 10% fetal or newborn calf serum, in a humidified atmosphere of 95% air, and 5% carbon dioxide at 37 degrees centigrade. Extracts of cells are analysed for metabolites, as well as for cyclic nucleotides and pyridine nucleotides, and for enzymes such as glycogen synthase and glycogen phosphorylase. All of the metabolites have been applied to measurements in whole brain, and are thus easily adapted for use with cells in culture.

Major Findings: We have employed several glial cell lines to characterize the rate of glycogen formation and breakdown, the activities of the regulatory enzymes, and the role of cyclic nucleotides in the process of glycogenolysis. Seven glial cell preparations are considered: (1) primary cultures of neonatal rat brains (RPA); (2) herpes virus transformed RPA cells, (HSV-RPA); (3) SV-40 transformed adult mouse brain astrocytes; (4) & (5) glia derived from methylnitrosourea induced tumors in situ (B9 & B82); and (6) & (7), Mills and Green cell lines, derived from human astrocytoma.

The RPA and HSV-RPA cell lines show essentially the same features of glycogen metabolism: the rate of glycogen synthesis is rapid after feeding, accumulating up to 200 nmoles/mg protein at 2.5 hrs, and thereafter the levels of glycogen decrease. The maximal level of glycogen is dependent on both the density of cultures, as well as the concentration of extracellular glucose. Active ("a") and inactive ("b") forms of both phosphorylase and synthase exist, and the ratios of a to b forms are comparable to reported values for rat brain. Agents that increase the levels of cAMP promote the typical features of glycogenolytic cascade: quantitative conversion of phosphorylase "b" to "a," followed by the rapid degradation of glycogen.

B9, B82, S22, Mills and Green cell lines all appear to be deficient in some form of glycogen synthesis. Phosphorylase activities for the B9, cell line are essentially similar to the HSV-RPA, but the active form of synthase is essentially absent, implying a defect in the synthase phosphatase system. The active form of synthase is also low in the B82 cell line and the levels of total phosphorylase are essentially absent suggesting a pleiotropic effect on glycogen synthesis in this mutant cell line. For the S22 cell line, glycogen synthase is essentially similar to the HSV-RPA. However, the phosphorylase is found entirely in the active form. Thus glycogen does not accumulate, because the rate of breakdown exceeds the rate of formation, implying a defect in phosphorylase phosphatase reaction.

Mills and Green cells both show a high resting level of glycogen that is substantially unaffected by refeeding. Levels of phosphorylase total

phosphorylase are sufficient to account for a substantial glycogenolysis, but the levels of phosphorylase "a" are low in both cell lines.

Cyclic Nucleotide metabolism: All astrocytic cell lines tested to date show an increase in cAMP after the administration of catecholamines. Primary astrocytes, and the Mills and Green cell lines show an elevation of cAMP in response to 2-Cl-adenosine. Other agents (histamine, serotonin etc.) are without effect.

In HSV-RPA cell lines, glycogenolysis appears mediated by a beta-catecholamine receptor system, since norepinephrine-induced glycogenolysis is blocked by alprenolol. Incubation with catecholamines cause a quantitative conversion of phosphorylase "b" to "a", and the rate of glycogenolysis is proportional to the dose of catecholamine.

Significance to Biomedical Research and the Program of the Institute:

A study of the regulation of metabolism in the brain is complicated by the presence of several cell types and the inability to determine the site of particular reactions. Such studies are facilitated by the use of primary as well as transformed cell lines in vitro. Although cell lines in culture may substantially differ from their counterparts in situ, they offer a first approach to the problems of regulation of metabolism of brain, which may be pursued in dissociated brain cells.

Since glial tumors in man are a major oncotype of brain tumors, an understanding of the metabolism of transformed glia may elucidate important mechanisms relevant to etiology and treatment. Comparisons of primary and transformed and primary astrocytes, and the experimental transformation of primary cultures may yield important information on the metabolic condition for, and consequences of the transformation process.

Several classes of glycogen deficient diseases have been well characterized in man, and an increasing number have been described in animals. Collection and characterization of cell lines mutated in specific loci of glycogen metabolism may be useful in the understanding of the biochemical genetics, underlying mechanisms, and possible treatment of individuals affected with glycogen deficient diseases.

Proposed Course of the Project. Investigation is continuing into the characterization of various cell lines deficient in glycogen synthesis. HSV-RPA cells have been mutagenized, and cloned, and various selection techniques are being attempted to yield cell clones deficient in glycogen synthesis and degradation. At present, the variety of mutant cell lines will permit cell hybridization studies between strains which are permissive, and non-permissive for glycogen synthesis.

Each cell line is being further characterized with respect to: cyclic nucleotide induced glycogenolysis, AMP regulation of phosphorylase "b," and activities of phosphorylase "b" kinase.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02142-06 LNC																				
PERIOD COVERED October 1, 1979 to September 30, 1980																						
TITLE OF PROJECT (80 characters or less) Cerebral Metabolism in Altered Metabolic States of the CNS																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">W.D. Lust</td> <td style="width: 35%;">Head, Sect. on Neurochem. Pharm.</td> <td style="width: 15%;">LNC NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>J.V. Passonneau</td> <td>Head, Sect. on Cellular Neurochem.</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>H. Arai</td> <td>Visiting Fellow</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>A. Wheaton</td> <td>Biol. Lab. Tech. (Micro).</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>G.K. Feussner</td> <td>Biol. Lab. Tech.</td> <td>LNC NINCDS</td> </tr> </table> <p style="margin-top: 20px;">(N.B. Project No. Z01 NS 02371-01 LNC entitled "Biochemical and Physiological Aspects of the Brain During Hypothermia and Hibernation" has been incorporated into this project.)</p>			PI:	W.D. Lust	Head, Sect. on Neurochem. Pharm.	LNC NINCDS	OTHER:	J.V. Passonneau	Head, Sect. on Cellular Neurochem.	LNC NINCDS		H. Arai	Visiting Fellow	LNC NINCDS		A. Wheaton	Biol. Lab. Tech. (Micro).	LNC NINCDS		G.K. Feussner	Biol. Lab. Tech.	LNC NINCDS
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COOPERATING UNITS (if any) None																						
LAB/BRANCH Laboratory of Neurochemistry																						
SECTION Section on Cellular Neurochemistry																						
INSTITUTE AND LOCATION NINCDS, NIA, Bethesda, Maryland 20205																						
TOTAL MANYEARS: <div style="text-align: center;">3.0</div>	PROFESSIONAL: <div style="text-align: center;">1.5</div>	OTHER: <div style="text-align: center;">1.5</div>																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords) <p>The effects of <u>ischemia</u> and recovery have previously been investigated in the <u>gerbil</u> cerebral cortex in vivo. The results have provided some evidence about the biochemical perturbations that could lead to irreversible brain damage. Of particular interest are the changes in <u>cyclic AMP</u>, the overshoots in <u>P-creatine</u> and <u>glycogen</u>, and the gradual restoration of the total adenylate pool. The significance of these changes to the survival of the animal are being examined in other regions of the brain that are known to be particularly susceptible to an ischemic episode. The increases in cyclic AMP both during and after ischemia can be mimicked in rat brain slices and this model is being used to identify the agonist(s) with specific receptor antagonists. Studies on the cerebral metabolism in brains from <u>hibernating hamsters</u> is being continued. The changes in metabolites including a reduction in cyclic GMP and an increase in GABA and lactate which appear to be directly related to hibernation have been demonstrated in other regions of the brain.</p>																						

Objectives: To examine the mechanisms involved in the ischemia-induced derangements of adenylates, cyclic AMP and glycogen. To determine if there is a biochemical explanation for the selective vulnerability of the CA-1 neurons of the hippocampus to an ischemic episode. To describe the biochemical changes that occur in the electrically quiescent hibernating brain and to distinguish these from the quiescent ischemic brain.

Methods Employed: Mongolian gerbils were anesthetized, the common carotid arteries were exposed and looped with suture. As the gerbils emerged from the anesthesia, the artery(ies) was ligated. Those animals which exhibited positive neurological signs were frozen at various times following ligation in liquid nitrogen.

The cerebral cortex was removed at -20° and extracted in perchloric acid. In the unilateral ischemia studies, the left hemisphere served as the ischemic side and the right as the control. ATP, P-creatine, glucose, glycogen, glutamate, citrate and GABA were determined enzymically. Cyclic nucleotides were measured by radio-immunoassay.

In the recovery studies, the carotid artery was occluded with an aneurysm clip for the appropriate time and then released. At various times following release, the gerbils were frozen and the brains removed as described above.

Brain slices. The rats were decapitated (zero-time of ischemia), the entire forebrain removed at 37° and the cerebral cortex was sliced and either fixed with perchloric acid (ischemia) or incubated in oxygenated phosphate-buffered saline containing glucose (recovery).

In situ fixation is a modification of the funnel-freezing technique first described by Kerr (1935). The conscious gerbil continued to breathe for up to 60 seconds as the brain was being frozen with liquid nitrogen. The advantages of this procedure are that oxygenation of the blood and cerebral circulation were maintained during the fixation process. By minimizing the fixation artefact, deeper regions of the brain could then be sampled.

Major Findings: The gerbil model of ischemia has been used to investigate the fate of certain energy metabolites, the cyclic nucleotides and some putative neurotransmitters during ischemia and recirculation. There are generally two types of response: 1) the rapid changes in the high-energy metabolites toward depletion and 2) the slower changes which include a rise in GABA and a fall in cyclic GMP and total adenylates. While the metabolite response during ischemia reflects the quiescence of the brain, the severity of the ischemia is not indicated.

The recovery phase following an ischemic insult represents a process that is substantially more complex than a mere reversal of the biochemical events which occurred during ischemia. During recirculation, there is a large secondary rise in cyclic AMP, an overshoot of glycogen, glucose and P-creatine and a relatively slow restoration of the adenylate pool. The expression of these events are generally more dramatic after longer periods of ischemia and

provide some evidence that certain metabolic abnormalities persist when other parameters indicate normalization of brain metabolism. Thus, it is quite possible that the recirculation period has its own set of pathogenic events which arise from the sudden availability of oxygen and glucose to a metabolically and functionally inactive tissue.

Brain Slices. Many of the biochemical events that occur both during and after an ischemic insult in vivo can be duplicated in vitro. Preliminary results indicate that the adenylate cyclase agonist responsible for the large post-ischemic rise in cyclic AMP does not appear to be adenosine, norepinephrine or histamine, but may be a combination of these and others as well. Identification of the agonist is of particular importance in determining the significance of cyclic AMP to the recovery process.

In another set of experiments, it was determined that both oxygen and glucose were needed for the regeneration of ATP. However, when only oxygen was added back to the system after 20 minutes of ischemia, there was a 15-fold increase in cyclic AMP with little or no detectible changes in the concentration of ATP. It is quite possible that there is a small compartment of ATP which serves as substrate for the adenylate cyclase as a number of other investigators have suggested.

Hibernation. Previous studies on the metabolite levels in the cerebellum and cerebral cortex suggested that the elevation of lactate, GABA and glucose and the depression of cyclic GMP were directly related to hibernation. Other differences could be attributed to fixation artefact. Subsequent studies on the metabolites in the corpus callosum, thalamus, hypothalamus, hippocampus, septum and caudate-putamen indicate that the changes in cyclic GMP and GABA observed in the cerebellum and cerebral cortex of brains from hibernating hamsters occur throughout the brain. However, the magnitude of the changes do vary with the different regions of the brain.

Significance to Biomedical Research and the Institute Program.

The gerbil model of ischemia in vivo has been particularly useful in defining the abnormal biochemical events which occur both during and after an ischemic event. If brain damage is related to a failure in the biochemical machinery, then further examination of these biochemical perturbations may provide some insight into the pathophysiological mechanisms that lead to brain damage. For example, the large post-ischemic rise in cyclic AMP may be critical to the restoration of brain function. It has been established by a number of investigators that cyclic AMP is inhibitory to the firing rates of certain neurons. It is quite possible that the elevated cyclic AMP could be a determining factor in the onset of electrical activity of the brain during this critical period. Until the cyclic AMP response can be manipulated by an appropriate cyclase blocker, the significance of cyclic AMP to the restoration of function, or the lack of it, really cannot be ascertained. Although many of the other metabolic abnormalities are not directly related to the excitability of the CNS, their impact on the recovery process could be just as

great. Normal levels of ATP cannot be regenerated as long as the concentrations of the total adenylates remain depressed. While the adenylates decrease slowly during ischemia, the recovery is also gradual and ATP can only be regenerated to about 90% of the total adenylates. Perhaps, increasing the adenylate pool size would minimize the ischemic brain damage.

The hibernating hamster is a good model for studying the transition of the brain from an active to an inactive state. A major difference in the cerebral metabolites between an ischemic and a hibernating brain, both in an electrically quiescent state, is that the energy state of the brain from hibernating hamsters is maintained. An elevation of GABA and a decrease in cyclic GMP occurs in both types of brains. Another factor is the large change in cyclic AMP in the ischemic brain which does not occur in the hibernating brain. The differences between these two quiescent brains may be useful in identifying those factors that lead to irreversible brain damage.

Proposed Course of Project: Previous experiments have emphasized the changes in metabolites that occurred in the cerebral cortex. By using sensitive quantitative histochemical methods, the biochemical response to ischemia will be determined in other discrete regions and eventually in single cells. This approach should provide some information relevant to the selective vulnerability of certain populations of cells to ischemia.

Since many of the pathophysiological events which occur in vivo can be reproduced in brain slices, this model can be used to examine the underlying mechanisms. In addition, brain slices may be useful for testing agents that might improve metabolic recovery.

Undoubtedly, many of the cellular responses to ischemia are invoked to preserve the viability of the tissue. It is the other processes that are incompatible with recovery that require attention. Modification of those events may be the first step to improving surviving following an ischemic episode.

Publications: Passonneau, J.V., Lust, W.D. and McCandless, D.W.: The preparation and analysis of biological samples for the measurement of metabolites. Techniques in Life Sciences. B2/11:1-27, 1979.

Conger, K.A., Garcia, J.H., Kauffman, F.C., Lust, W.D., Murakami, N. and Passonneau, J.V.: Alanine to glutamate ratios as an index of reversibility of cerebral ischemia in gerbils. Ann. Neurol. (in press).

Lust, W.D., Murakami, N., de Azeredo, F. and Passonneau, J.V.: A comparison of methods for brain fixation. In: Passonneau, J.V., Hawkins, R., Lust, W.D. and Welsh, F., (Eds.): Cerebral Metabolism and Neural Function. Baltimore, Williams and Wilkins, (in press).

Murakami, N., Lust, W.D., de Azeredo, F., and Passonneau, J.V.: Ischemia-related changes in adenine nucleotide metabolism. In: Mrsulja, B.B., Rakic, Lj. M., Spatz, M. and Lust, W.D., (Eds.): The Pathophysiology of Cerebral Metabolism. New York, Plenum Press, (in press).

Project No. Z01 NS 02371-01 LNC entitled "Biochemical and Physiological Aspects of the Brain During Hypothermia and Hibernation" has been incorporated into this project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02254-04 LNC
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PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Use of Neurological Grafts to Repair the Injured Peripheral or Central Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. A. Zalewski LNC NINCDS

Other: A. K. Gulati LNC NINCDS

COOPERATING UNITS (if any)

W. K. Silvers, Department of Human Genetics, University of Pennsylvania

LAB/BRANCH

Laboratory of Neurochemistry

SECTION

Neuronal Development and Regeneration

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.7

PROFESSIONAL:

1.8

OTHER:

0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINDRS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previously it was demonstrated that host nerve fibers could successfully regenerate through a 4 cm length of nerve allograft that contained minor antigens provided that the host rat was rendered immunologically tolerant to the transplantation antigens of the nerve donor. A similar result has presently been obtained in tolerant rats grafted with nerves that bore the more highly antigenic major as well as minor antigens. However, after tolerance was abolished (by injecting lymphoid cells already sensitized to allograft antigens) in rats with successful nerve allografts, rejection occurred and host nerve fibers in the graft degenerated and disappeared. Although tolerance induction is an experimental model of immunosuppression, the results indicate that the prevention of rejection is the main barrier to overcome when allografts are needed to repair injured peripheral nerve. An evaluation of currently available immunosuppressive drugs has revealed that Cyclosporin-A is the most effective in preventing nervous tissue rejection.

Project Description:

Objective: The purpose of this project is to determine the extent to which a peripheral nerve allograft (a graft between genetically different members of the same species) can be used to aid in the repair of injured nerve tissue. It has been recognized that transplantation antigens on the cells in a nerve allograft evoke an immune reaction by the host and that this reaction may prevent host nerve fiber regeneration through the graft. While host nerve fibers can grow through short-lengths of nerve allografts, it is rare for fibers to regenerate through longer lengths (4 cm) even though the antigenicity of the allograft is purportedly reduced by freezing or irradiation. It would appear then that host immunosuppression offers the best way to promote regeneration through a long-length of nerve allograft. Since there have been few studies in which drug immunosuppression for nerve allografts has proved successful, the initial step in evaluating nerve allografts was to develop a reliable method of host immunosuppression. This was done by utilizing animals that were immunosuppressed by rendering them neonatally tolerant to the transplantation antigens of the nerve graft donor. Briefly, the procedure for obtaining tolerance is to inject appropriate doses of adult lymphoid cells from one rat strain into neonatal rats from another strain. Because the immune system of the neonatal rat is immature, it responds to the inoculated allogenic cells by developing suppressor lymphocytes and antibody which prevents the rejection of the inoculated cells. The tolerance that develops toward foreign lymphoid cells is permanent and extends to other tissue grafts because lymphoid cells express all the antigens that are present in other tissues. Thus, once the inoculated neonatal rat matures it can be grafted with tissues like skin or nerve, which in turn will survive indefinitely, provided that these allografts originate from a donor of the same genotype as the tolerizing lymphoid cells. As expected, host nerve fibers of tolerant rats were able to regenerate through a long nerve allograft and reinnervate denervated muscles. However, because the rat strains used in the previous tolerant study differed only in minor antigens, it is essential to determine whether similar regeneration can occur in tolerant rat grafted with nerves containing the more highly antigenic major as well as minor transplantation antigens. In addition, another important question that needs to be answered is what will happen to host nerve fibers in a nerve allograft if subsequently the allograft is now rejected. Tolerance can be abolished by injecting lymphocytes that are sensitized to the antigens of the nerve allograft. The sensitized cells can be obtained from a normal rat who has rejected an allograft of the same genotype as that present in the tolerant rat. Finally, since the tolerant model has demonstrated that rejection is the main barrier to overcome in using nerve allografts, an evaluation of currently available immunosuppressive drugs, particularly the new one Cyclosporin-A, was initiated.

Methods Employed:

Inbred Lewis (LE) and Brown Norway (BN) rats were used. These rats differ in antigens derived from the major and multiple minor transplantation loci of the species. LE rats were neonatally made tolerant to BN antigens by treatment with (LE x BN) F_1 hybrid bone marrow cells. When the bone marrow-

treated or normal LE rats were 4-6 months old, a segment of peroneal nerve was removed from their thighs which resulted in the denervation of the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. A 4 cm long graft of BN nerve was then inserted between the injured nerve ends of the LE rats in order to determine whether the nerve graft might provide a pathway through which host nerve fibers might regenerate and ultimately grow to re-innervate the denervated TA and EDL muscles. Some tolerant but not normal rats were bilaterally grafted with a 4 cm BN (right leg) or LE (left leg) nerve. Four months later these rats were injected intraperitoneally with a suspension of LE lymphoid cells that were sensitized to BN antigen. Rejection of the BN but not LE nerve graft would now be expected to occur after this treatment because sensitized lymphocytes can overcome the protection provided by suppressor lymphocytes and antibody. The findings after abolishing tolerance might be equated to what might happen to allograft survival after successful drug immunosuppression was stopped since sensitized cells would be expected to appear. The new immunosuppressive drug Cyclosporin-A (CsA) was used (25 mg/kg/ subcutaneous) to see if it could prevent the rejection of ganglia, nerve and muscle allografts. This agent was chosen since earlier studies indicated it was superior to other drugs.

Major Findings: LE nerve fibers were able to regenerate through 4 cm BN nerve grafts in tolerant but not normal LE hosts. Moreover, the regenerated LE axons were successful in restoring innervation to the denervated TA and EDL muscles. However, after tolerance was abolished, rejection of BN but not LE nerve grafts occurred and host nerve fibers in the allograft degenerated and disappeared. Histological examination revealed that the BN nerve graft was intensely infiltrated by mononuclear cells which were absent from the LE graft that was present in the opposite thigh of tolerance-abolished rats. CsA was effective in preventing the rejection of BN grafts by normal LE rats. CsA treatment was carried out for only 30 days since the supply of this new drug was limited. Nevertheless, the CsA results showed that: (1) the cellular immune reaction to the nerve allograft was prevented, (2) that host axons had grown about 4 cm into the nerve allograft, and (3) that the drug was not toxic (i.e., no weight loss, lethargy or hematological cell changes occurred).

Significance: The present findings show that host nerve fibers can regenerate through long, highly antigenic nerve allografts and reestablish functional neuromuscular connection but only in immunosuppressed hosts. This result clearly demonstrates that the prevention of rejection is the main barrier to overcome when allografts are needed to aid in the repair of injured nerve. However, the observation that host axons in the allograft degenerated after immunosuppression was terminated by abolishing tolerance is a cause for concern since tissues again become denervated. Whether a second wave of host axonal regeneration will occur through the connective tissue remnants of the allograft is currently being investigated. The immunosuppressive drug CsA was shown to be effective in preventing nerve allograft rejection and allowing host axons to regenerate into the graft. Further studies with CsA are needed because by comparison it is superior to other immunosuppressive agents. An additional supply of CsA has recently been obtained and its long-term use will be evaluated. It will be interesting to see whether

after successful regeneration, allograft rejection occurs after cessation of CsA.

Proposed Course of Project:

- (1) Repeat tolerant study to determine if after abolishing tolerance host axons can regenerate for a second time through the remnants of a rejected nerve allograft.
- (2) Perform a long-term study with CsA to determine if successful regeneration occurs through a long allograft (grafts with major and minor or only minor antigens will be tested). If successful, CsA will be withdrawn to see if allograft rejection occurs and what happens to host axons in the allograft.
- (3) A study will be performed to determine whether nerve allografts of various lengths are effective in aiding regeneration in a sensitized host. Usually a sensitized host rejects an allograft faster and because of this it might be that none or only very short grafts will prove successful. Since blood transfusions are the prime means of inducing sensitization, hosts will be sensitized in that way. On the other hand, current evidence suggests that blood may have immunosuppressive qualities especially when multiple, spaced transfusions are given. For this reason, it is pertinent to evaluate blood alone or combined with drugs for immunosuppression of nerve allografts.
- (4) A series of studies will be performed to determine whether altering allograft antigenicity affects host nerve regeneration. It is known that leukocytes in a graft greatly contribute to allograft antigenicity. Leukocytes could be removed from grafts by treating the host or graft with: (1) irradiation (leukocytes are sensitive to radiation dosages that do not alter Schwann cells), or (2) anti-lymphocyte serum (this kills leukocytes present in the graft). In addition, nerve allografts will be used after various periods of tissue culture since cells in culture may lose antigens or present them in lesser antigenic forms. Experiments will also be performed to reevaluate why frozen nerves fail to promote host nerve regeneration.
- (5) Experiments will be done to evaluate nerve allograft length and nerve allograft dosage as factors which influence nerve repair. For example, if two nerves need repair what happens if one requires a 1-cm allograft while the other needs 5 cm. Will one or both repairs fail?

Publications:

- (1) Zalewski, A. A., and W. K. Silvers. An evaluation of nerve repair with nerve allografts in normal and immunologically tolerant rats. J. Neurosurg. 52: 557-563, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02256-04 LNC
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Metabolic Profiles in Normal and Diseased Retina		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J.V. Passonneau Head, Sect. on Cellular Neurochem. LNC NINCDS OTHER: W.D. Lust Head, Sect. on Neurochem. Pharm. LNC NINCDS		
COOPERATING UNITS (if any) E.K. Barbehenn LVR, NEI		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Section on Cellular Neurochemistry		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Studies are in progress on <u>retinal metabolism</u> in frozen sections of retinal layers. The effects of <u>dark-and light-adaptation</u> on high-energy phosphate compounds and cyclic nucleotides are being investigated. ATP, P-creatine, cyclic GMP, cyclic AMP, GTP, and GDP have been measured in 8 retinal layers dark-adapted frogs, and after 2 min or 2 hr of light exposure. In addition the enzyme, guanylate cyclase has been measured under the same conditions.		

Project Description:

Objectives: To determine the distribution of metabolites, putative transmitters, cyclic nucleotides in the layers of frozen-dried frog retina in normal animals and animals treated with drugs to destroy the rod outer segments. To study retina metabolism during light and dark adaptation, and metabolic changes during the shedding process of the rod outer segments. Future plans include the study of pathological states such as diabetic retinopathy and gyrate atrophy. Comparable techniques will be used for the study of pathological conditions.

Methods Employed: In the studies on retinal layers, frogs are decapitated into liquid nitrogen, the heads frozen and stored at -70° until dissection. The eyes are dissected at -20° and the whole eyeball removed. The eyeball is mounted on a specially designed holder, and sectioned at $10\text{ }\mu\text{m}$ at -25° in a cryostat. The sections are vacuum-dried at -40° , after which they can be stored at 20° under vacuum. For microdissection, the samples are brought to room temperature under vacuum, removed, and the retinal layers (approx. $0.1\text{ }\mu\text{g}$) dissected with microinstruments using a low-power dissecting scope. The microchemical analyses are performed in oil wells using volumes of 0.05 to $0.05\text{ }\mu\text{l}$.

Major Findings: ATP and P-creatine showed an increasing concentration gradient from the outer segments to the inner layers; both compounds are three- to four-fold higher in the inner retina than in the photoreceptor cells. After 2 min of illumination in vivo both ATP and P-creatine increased. The changes were most marked in the photoreceptor portion, the sum of ATP and P-creatine increased almost 2-fold. The increase in energy reserves in light supports the hypothesis that light is the absence of stimulus and that transmitter release and the concomitant energy expenditure occur primarily in the dark.

Cyclic GMP Preliminary experiments indicated that cyclic GMP decreased in the photoreceptor cells, but not in the inner retina following exposure to light. In the detailed study of the layers, cyclic GMP was found to be in highest concentrations in the outer segments of frog retina, and was 40-fold lower in the inner retina. After 2 min exposure to light, the cyclic GMP in the photoreceptor cells, decreased in to 1/6 the dark values. There was a 5-fold increase in the outer plexiform layer and a 14-fold increase in the inner nuclear layer. After 2 hours of light, cyclic GMP remained decreased in the photoreceptor cells, while in the remaining retina the values were like those of dark adapted retina. The concentrations of GDP were uniformly distributed among the retinal layers, whereas GTP was highest in the outer nuclear layer. After exposure to light, GTP decreased and GDP increased in all layers. The decrease of GTP in contrast to ATP, implies another function, such as the phosphorylation of proteins.

The guanylate cyclase was found to be largely in the photoreceptor portion of the frog retina. The activity of guanylate cyclase increased after 2 min or 2 hr of illumination. This in contrast to the changes found in vitro.

Cyclic AMP did not change significantly in any of the layers after light exposure. The decrease in cyclic GMP after light exposure, accompanied by an increase in guanylate cyclase activity and a decrease in the substrate GTP implies an enormous turnover rate for the cyclic nucleotide. Such an rapid turnover would in turn suggest that the cyclic GMP could function as a chemical transducer in the visual response.

Significance to Biomedical Research and the Program of the Institute:

The layered structure of the retina makes possible the location of certain metabolites within parts of cells, such as outer rods and cones and the nucleated region of the photoreceptor cells. The identification of the site of enzymes, and metabolites will help elucidate metabolic controls involved in the visual process.

The function of cyclic GMP in the retina is not yet understood, although the high concentrations and the changes during light- and dark-adaptation imply a role in the transduction of the visual response. The techniques in our laboratory provide a unique possibility to study the effects of light in vivo, or the changes which might occur in retinopathies.

Proposed Course of Project: The preliminary work in retinal layers will be developed further. Both normal and diseased retinas will be studied in an attempt to learn the biochemical nature of retinopathies.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02257-04 LNC												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Neuropharmacology of Cerebral Metabolism (Former Title: Biochemistry of Experimental Seizures)														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: W.D. Lust</td> <td style="width: 40%;">Head, Sect. on Neurochem. Pharm.</td> <td style="width: 10%;">LNC</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td>OTHER: J.V. Passonneau</td> <td>Head, Sect. on Cellular Neurochem.</td> <td>LNC</td> <td>NINCDS</td> </tr> <tr> <td>A.B. Wheaton</td> <td>Biol. Lab. Tech. (Micro.)</td> <td>LNC</td> <td>NINCDS</td> </tr> </table> (N.B. Project No. Z01 NS 01942-08 LNC entitled "The Role of Cyclic AMP and Cyclic GMP in the Central Nervous System" has been incorporated into this project.)			PI: W.D. Lust	Head, Sect. on Neurochem. Pharm.	LNC	NINCDS	OTHER: J.V. Passonneau	Head, Sect. on Cellular Neurochem.	LNC	NINCDS	A.B. Wheaton	Biol. Lab. Tech. (Micro.)	LNC	NINCDS
PI: W.D. Lust	Head, Sect. on Neurochem. Pharm.	LNC	NINCDS											
OTHER: J.V. Passonneau	Head, Sect. on Cellular Neurochem.	LNC	NINCDS											
A.B. Wheaton	Biol. Lab. Tech. (Micro.)	LNC	NINCDS											
COOPERATING UNITS (if any) Pharmacology Laboratory, Epilepsy Branch														
LAB/BRANCH Laboratory of Neurochemistry														
SECTION Section on Cellular Neurochemistry														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: 1.1	PROFESSIONAL: 0.6	OTHER: 0.5												
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>														
SUMMARY OF WORK (200 words or less - underline keywords) It is increasingly evident that 1) the rate of <u>energy utilization</u> is coupled to CNS function and 2) that the <u>cyclic nucleotides</u> as neuroeffectors may play an important role in neuronal excitability. Using the levels of both energy metabolites and cyclic nucleotides as an indicator of brain activity, the effect of several neurotropic agents including 1) <u>convulsants</u> , 2) <u>anticonvulsants</u> and 3) <u>anesthetics</u> were examined in the cerebral cortex and cerebellum. Generally, these drugs have little or no effect on the concentrations of the energy metabolites. However, with the onset of <u>seizures</u> following treatment with convulsants, there was a decline in the energy status of the tissue. While the levels of cyclic AMP increased during the tonic extensor phase of convulsions, it was not generally responsive to pharmacological manipulation. In contrast, cyclic GMP levels in the cerebellum were depressed by convulsants and anesthetics and elevated by convulsants. Although cyclic GMP in the cerebral cortex was similarly affected, the changes were not as dramatic.														

Objectives: a) To determine what effect the different groups of neurotropic agents have on energy metabolism and on cyclic nucleotide metabolism.

b) To investigate cerebral metabolism during experimental seizures and to relate the biochemical events to the initiation, propagation and termination of seizures.

Methods employed: Animals were rapidly frozen either intact or in situ with liquid nitrogen at the appropriate times after treatment. The brains were removed at -25° , weighed and extracted in perchloric acid. The neutralized extracts were used in all subsequent assays. When discrete regions of the brain were examined, the tissue was sectioned at -20° , dried at -45° , and the brain area free-hand dissected at room temperature. The tissue was weighed on a quartz-fiber balance and extracted in either sodium hydroxide or hydrochloric acid depending on the metabolite to be measured.

The cyclic nucleotides were measured by radioimmunoassay in the whole as well as in the discrete regions. The energy metabolites for the whole regions were determined by direct enzymic analysis, but in the discrete regions required the use of enzymic cycling.

Maximal electroshock was applied by corneal electrodes at an intensity of 50 mA for 0.2 sec. The electroshock produces a convulsive behavior manifested by a) tonic extension from 2 to 13 sec, b) an intermittent clonic phase from 13 to 25 sec and c) a postictal depressive phase thereafter.

Major Findings: Experimental seizures. The studies on MES clearly demonstrated that during the excitable stages of convulsion there was a pronounced decrease in the energy status and an increase in the cyclic nucleotides in the brain regions examined. While the changes in the energy metabolites and cyclic GMP were similar in both the cerebellum and cerebral cortex, the elevation of cyclic AMP in the cerebral cortex was both greater and of longer duration than in the cerebellum. Studies on single cells from both regions indicate that the greater amount of energy reserves in the cerebellum may account for the attenuated cyclic AMP response in this region after MES.

Pretreatment of mice with phenytoin blocked the tonic extension following MES and also diminished the changes in the metabolites in the cerebellum but not in the cerebral cortex. The evidence indicates that the cerebellum may be involved in the anti-tonic extension effect of phenytoin.

To determine the significance of the metabolite changes described above to the events leading up to and triggering a convulsion, chemically-induced seizures were examined in mice with an emphasis on the preconvulsive and the triggering stages. Following treatment with the convulsant isoniazid, only cyclic GMP exhibited a change in the preconvulsive stage. The increase in cyclic GMP was evident also after the onset of the seizure, as was an in-

crease in the levels of cyclic AMP. In the early stages of the seizure, there was little or no change in the energy metabolites. Thus, it would appear that alterations in cyclic AMP and the energy metabolites are due to the seizures, whereas the changes in cyclic GMP may be related to seizure susceptibility.

Generally, anticonvulsants have little effect on the cerebral metabolites, but do depress the cerebellar levels of cyclic GMP. When anticonvulsants are given in combination with convulsants, the preconvulsive rise in cyclic GMP is prevented. Of interest is that of all the anticonvulsants only valproate has an effect on cyclic GMP in the cerebral cortex, a 50% increase. Thus, both valproate and isoniazid alone elevated the cyclic GMP in the cerebral cortex; however, in combination the levels are not significantly different from those of control.

Anesthetics: Dose response. The effect of halothane on the cerebral metabolites was examined in the cerebellum, cerebral cortex and spinal cord. Of the energy metabolites, only lactate was slightly elevated at higher concentrations of halothane which may indicate an hypoxic component. With increasing levels of halothane, cyclic GMP was elevated in the cerebral cortex and essentially depleted in the cerebellum. Cyclic AMP was only slightly depressed and only in the cerebral cortex.

Time course. At a concentration of 1.25% halothane, the cerebral metabolites were measured over a period of 4 hours. The results were consistent with those for the dose response; however, the cyclic GMP levels in the cerebral cortex did not remain elevated. It would appear that the halothane-induced rise in cyclic GMP is unrelated to its anesthetic action, but represents a side effect of the drug.

Significance to Biomedical Research and the Institute Program

A convulsion is a symptom of an underlying abnormality in brain function. Efforts to identify the cause of the spontaneous uncontrolled discharge of the neurons have indicated that the process leading up to and triggering seizure is quite complex. Besides evaluating the changes that reduce seizure threshold, other events occurring during the propagation and termination of the convulsion are of interest, since these processes may be equally important in the control of seizures.

The evidence to date indicate that of all the metabolites measured only cyclic GMP and GABA appear to play a role in the onset of seizures. The depletion of high-energy metabolites and the increase of cyclic AMP seem to be a result of seizure activity. While this tends to rule out their involvement in seizure susceptibility, it is quite possible that these changes play a role in the termination of overt seizures. The significance of the cyclic GMP to the triggering of seizures is supported by the finding that anticonvulsants generally prevent the preconvulsive rise in cyclic GMP as well as the seizure itself. Pharmacological manipulation of the cyclic GMP system may be a useful criteria for evaluating therapeutic efficacy.

Of particular interest is the role of cyclic AMP in the seizure process. Generally, the levels of cyclic AMP remained unchanged unless energy metabolism is compromised (i.e., a fall in high-energy phosphates). It would appear that the cyclic AMP system is only invoked when all other systems have failed and is an attempt of the brain to shut down until the deleterious stimulus has disappeared. The apparent relationship between energy metabolism and cyclic AMP may be mediated by adenosine. Since the cyclic AMP is relatively insensitive to mild alterations in CNS activity, it is unlikely that cyclic AMP metabolism will be susceptible site for pharmacological intervention and therefore be useful in the control of seizures.

Elevation of cyclic GMP in the cerebral cortex is a common occurrence after CNS stimulation (i.e., seizures). Why halothane at anesthetic doses produces a similar rise in cyclic GMP is presently unclear, but may be related to an action of the drug other than anesthesia.

Proposed Course of Project: Realizing some of the deficiencies in the seizure models used, the kindling of rats with the electrodes placed in the amygdala is being undertaken in collaboration with Drs. Kupferberg and Sato. The emphasis will be placed on the pre-kindling and interictal phases as well as the seizure itself. An advantage of this study over previous ones will be that implanted electrodes will be used to monitor EEG activity.

In subsequent investigations on neurotropic drugs as well as seizures, a greater emphasis will be placed on the discrete sampling of tissue. It has been our experience that a number of phenomenon only became evident after microsampling.

Publications: Lust, W.D., Feussner, G.K., Passonneau, J.V. and McCandless, D.W.: The effect of anticonvulsants on cyclic nucleotide metabolism in brain. In: Palmer, G. (Ed.): Neuropharmacology of Central and Behavioral Disorders, New York, Academic Press, (in press).

Lust, W.D., Feussner, G.K., Passonneau, J.V. and McCandless, D.W.: Biochemical evidence for a role of the cerebellum in the control of seizures. Fed. Proc. (invited manuscript).

Project No. Z01 NS 01942-08 LNC entitled "The Role of Cyclic AMP and Cyclic GMP in the Central Nervous System" has been incorporated into this project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02429-01- LNC
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Coordinate Effects of Amphetamine on Brain Energy Metabolism and Protein Synthesis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J.V. Passonneau Head, Sect. on Cellular Neurochem. LNC NINCDS Other: T.S. Nowak, Jr. Staff Fellow LNC NINCDS		
COOPERATING UNITS (if any) none		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Section on Cellular Neurochemistry		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.4	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Effects of <u>amphetamine</u> on <u>brain energy metabolism</u> and <u>protein synthesis</u> are being investigated under conditions which give rise to changes in <u>body temperature</u> in response to the drug. In particular, metabolic changes associated with drug-induced <u>hyperthermia</u> are being correlated with the hyperthermia-dependent <u>inhibition of brain protein synthesis</u> by amphetamine. Effects on protein synthesis are determined by means of brain <u>polyribosome profiles</u> . Metabolites of interest include brain <u>glucose</u> , <u>glycogen</u> and <u>high-energy phosphates</u> , especially <u>guanine nucleotides</u> . Methods for the assay of GTP, GDP and GMP have been improved.		

Project Description:

Objectives: To determine whether known effects of amphetamine on brain energy metabolism (e.g. increased glycogen breakdown) are correlated with hyperthermia produced by the drug. To characterize other possible changes in brain metabolites, notably guanine nucleotides, during amphetamine-induced hyperthermia. To examine the link between known and postulated effects of amphetamine on brain metabolites with the demonstrated inhibition of brain protein synthesis by amphetamine, and thereby to elucidate possible biochemical control mechanisms by which energy status regulates protein synthesis.

Methods Employed: d-Amphetamine sulfate is administered by intra-peritoneal injection to mice housed at various temperatures. (An ambient temperature of 27°C is used in routine studies requiring amphetamine-induced hyperthermia). At appropriate times after injection the mice are frozen rapidly in liquid nitrogen, and samples of cerebral and cerebellar cortex are assayed for glucose, glycogen, adenine and guanine nucleotides, and other metabolites using enzymatic assay methods. Polyribosomes are prepared from the remaining brain tissue, which are further fractionated on sucrose gradients to yield "polyribosome profiles" as an index of brain protein synthesis.

Major Findings: In the initial stages of this project it has been demonstrated that in the mouse, as in the rat, the effect of amphetamine on body temperature is dependent on the ambient temperature at which the animals are housed. At 20°C, NIH general purpose mice show only slight elevation of body temperature with up to 20 mg/kg amphetamine, while at 27°C pronounced hyperthermia is evident with even 5 mg/kg. These initial observations will now allow the comparison of amphetamine effects on brain metabolites both with and without concomitant hyperthermia.

Significance to Biomedical Research and the Program of the Institute: These studies should provide information about the functional, physiological role of catecholamine pathways in the brain, as characterized by their sensitivity to the pharmacological actions of amphetamine. On a more fundamental biochemical level, they should also help to characterize the control mechanisms by which energy metabolism must be linked with protein synthesis and other energy-utilizing cellular processes.

Proposed Course of Project:

Work to date has involved working up assay methods and characterizing the experimental system. Amphetamine effects on brain metabolites and protein synthesis can now be compared under conditions which either allow or fail to allow a hyperthermic response to the drug.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02430-01- LNC												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Aspects of Calcium Metabolism in Electric Tissue														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">R. W. Albers</td> <td style="width: 15%;">LNC</td> <td style="width: 35%;">NINCDS</td> </tr> <tr> <td></td> <td>S. P. Chock</td> <td>LNC</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>L. M. Amende</td> <td>LNC</td> <td>NINCDS</td> </tr> </table>			PI:	R. W. Albers	LNC	NINCDS		S. P. Chock	LNC	NINCDS		L. M. Amende	LNC	NINCDS
PI:	R. W. Albers	LNC	NINCDS											
	S. P. Chock	LNC	NINCDS											
	L. M. Amende	LNC	NINCDS											
COOPERATING UNITS (if any) None														
LAB/BRANCH Laboratory of Neurochemistry														
SECTION Section on Enzyme Chemistry														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: 1.1	PROFESSIONAL: 0.9	OTHER: 0.2												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) <p>Regulatory roles of <u>calcium</u> in Electrophorus electric organ tissue function are under investigation. At present the studies consist of two parts: (a) the interaction of <u>calmodulin</u> with <u>electroplaque membranes</u>; and (b) characterization of a Ca^{++}-dependent ATPase associated with electroplaque membranes.</p>														

Project Description:

Objective: These studies are designed to assess the role of Ca^{++} in the function of a highly specialized cholinergic tissue exemplified by the Electrophorus electric organ.

Methods Employed:

a) Calmodulin is assayed by a coupled enzyme method that is based upon the activation of brain c-AMP phosphodiesterase by calmodulin. Membrane fractions are prepared by differential centrifugation. Techniques are under development for removal of membrane-bound calmodulin. b) Ca^{++} -dependent ATPase is measured by an enzyme-coupled spectrophotometric assay or by a radiometric method. Standard steady-state kinetic analyses are applied.

Major Results: a) Membrane fractions of electroplaque tissue have been shown to contain bound calmodulin. Methods have been developed to dissociate this calmodulin. The calmodulin-free membranes can reassociate with exogenous calmodulin in the presence of Ca^{++} . b) A significant level of Ca^{++} -dependent Mg^{++} -ATPase activity is associated with electroplaque membranes. This activity can be solubilized by certain detergents.

Proposed Course: We plan to characterize the component of electroplaque membranes that bind calmodulin. Because the $(\text{Ca}^{++} + \text{Mg}^{++})$ -ATPase of red cells and of brain are activated by calmodulin, it may be that this ATPase in electroplaque is also calmodulin dependent. We will attempt to purify the calmodulin-binding component from the membranes. If it can be sufficiently purified it may be useful to prepare antibody for use in sub-cellular localization. In particular one would like to determine whether the calmodulin interactions are primarily with the innervated, non-innervated, or presynaptic membranes.

Significance: Calmodulin is a ubiquitously distributed protein that is considered to mediate many, if not most, intracellular actions of calcium ions. Electric organ tissue contains calmodulin in unusually high levels. Because of the clearly defined function of this tissue as a cholinergic generator of electrical discharges, electric organ provides a unique opportunity to examine the role of calmodulin in this function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02255-04 LNC
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) GABA and Nucleotide Metabolism in Brain Mitochondria		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: R. W. Albers LNC, NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Enzyme Chemistry		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated and the following paper has been published: Lopez-Cardozo, M. and Albers, R.W.: Relationship between the 4-aminobutyrate bypath and the oxidation of 2-oxoglutarate in rat brain mitochondria. <u>J. Neurochem.</u> 33: 1259-1265, 1979.		

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Molecular Biology
National Institute of Neurological and Communicative Disorders and Stroke

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Another stage of viral development at which one could interfere with viral multiplication is the replication of the viral genome and its assembly into a complete virus. While it is known that the replication requires the simultaneous synthesis of nucleocapsid proteins, many attempts to achieve it in vitro have failed. Therefore, a permeabilization method of mammalian cells with lysolecithin has been developed which allows nucleoside triphosphates and other compounds to enter the cells. The resulting system closely resembles an infected cell in vivo, but the components needed for viral replication and assembly can be added to or left out of the medium at will.

2. Mechanisms controlling microbial differentiation (sporulation).
Microbial differentiation generally starts when rapidly metabolizable carbon or nitrogen sources or phosphate are used up. This is true for both prokaryotes, such as Bacillus subtilis, and eukaryotes, such as the yeast Saccharomyces cerevisiae whose cells have to undergo meiosis before the resulting haploid cells can produce spores. A similar nutritional control of differentiation exists also in higher organisms but it has not been studied in any detail. We are convinced that understanding of differentiation in microorganisms will greatly accelerate the understanding of embryonic differentiation in higher organisms. The Laboratory's studies of sporulation in B. subtilis have utilized conditions of excess glucose, ammonium ions and phosphate, so that differentiation can never start by itself. Many mutants and inhibitors were investigated for the ability to initiate sporulation; only conditions causing a partial deprivation of purine nucleotide synthesis (in particular of guanine nucleotides) were found to initiate sporulation. It was essential to reduce the synthesis of GTP only partially so that the GTP concentration inside the cell decreased below a critical level. Too much inhibition simply arrested all metabolism, including that needed for differentiation. The B. subtilis system appears one of the simplest systems in the evolution of cellular differentiation because it does not require the production of any special compound such as a hormone to initiate differentiation but controls it by the nucleotide GTP (or GDP).

Normal nutritional deprivation can lead to differentiation by several mechanisms. Phosphate starvation directly causes a deficiency of nucleotides. Nitrogen starvation causes a deficiency of glutamine which is needed for purine nucleotide synthesis, and a starvation of carbon compounds decreases P-ribosyl-PP which is required for nucleotide synthesis. In addition to these three simple mechanisms, bacteria have developed a more sophisticated method to initiate differentiation when one or more amino acids are rapidly depleted. This was shown by media changes causing the sudden lack of amino acids or by mutants, auxotroph for an amino acid and lacking the active transport mechanism for it. Amino acid starvation initiated the stringent response, whereby RNA synthesis is arrested and the highly phosphorylated nucleotides ppGpp and pppGpp are produced in increased quantities. As a result the concentration of GTP decreased. Again, partial but not complete deprivation of amino acid supply allowed sporulation. This process was prevented in a "relaxed" mutant in which the stringent response is abolished. The result showed that the stringent response and the production of the highly phosphorylated nucleotides acted so to say as an intracellular hormone, causing a decrease of GTP and thereby sporulation. However, even in the

relaxed mutant, sporulation could still be induced by compounds (e.g., decoyinine) causing a decrease of GTP without a concomitant increase of ppGpp. The results showed furthermore that GTP had to decrease below a threshold level in order to initiate the developmental processes. The need for this delicate metabolic balance explains why so many compounds or mutations can interfere with differentiation in B. subtilis and by inference in higher organisms. To assure proper differentiation, cells have evolved many mechanisms that control the concentration of nucleotides by synthetic and degradative enzymes. These mechanisms are now under investigation.

The possibility to induce differentiation by specifically reducing the synthesis of guanine nucleotides makes it feasible to distinguish between cellular reactions that are necessary for sporulation and others that also occur at the end of growth when sporulation usually starts. It has been shown that enzymes of the citric acid cycle or certain gluconeogenic processes are not necessary for differentiation when it is initiated in the presence of glucose. Similarly, manganese, required as co-factor for a number of enzymes, in particular phosphoglycerate mutase, and which was thought to be absolutely necessary for sporulation, is no longer required if sporulation is initiated by guanine starvation. This differentiation process is also not controlled by the same mechanism as catabolite repressible enzymes because guanine starvation which initiates sporulation does not prevent catabolite repression. However, other processes normally observed during differentiation still take place following guanine deprivation. For example, extensive protein turnover is initiated and aspartate transcarbamylase decreases within fifteen minutes of guanine starvation. Most interestingly, guanine starvation causes a rapid cessation of rRNA synthesis, a phenomenon that was previously associated only with the stringent response; i.e., the appearance of ppGpp. This phenomenon is now under investigation.

Similar studies of differentiation in the yeast, Saccharomyces cerevisiae, have been much more difficult because surprisingly little is known about many metabolic reactions although yeast is the most extensively studied eukaryotic cell. For example, it was not understood why cells grown in glucose are, after extensive washing, unable to adapt to growth on acetate as sole carbon source although they can adapt to ethanol, which is metabolized via acetate. This is not explained by the finding that gluconeogenic enzymes are repressed during growth on glucose because they have to be synthesized to allow growth on either acetate or ethanol. The essential reason for this difference was shown to be the lack of ATP which is rapidly consumed by the conversion of acetate to acetyl-Co-A whereas it can be regenerated if ethanol is metabolized via alcohol dehydrogenase and aldehyde dehydrogenase, since both reactions reduce NAD to NADH which enters electrons into the electron transport system. Sporulation in yeast has so far been studied exclusively by the transfer of cells to potassium acetate, generating under conditions of nitrogen starvation. The laboratory is now establishing conditions under which sporulation can be initiated also by carbon limitation because it can then determine biochemically and by the use of mutants and inhibitors which cellular metabolite has to decrease to allow the initiation of meiosis and sporulation.

3. Control of the synthesis of beta-adrenergic receptors and their coupling to adenylate cyclase. It had previously been reported by this Laboratory that exposure of HeLa cells to butyrate produces an increased response of cAMP formation to compounds such as isoproterenol which bind to and activate B-adrenergic receptors. Whereas this overall response was observed at butyrate concentrations of about 5 mM, the number of B-adrenergic receptors increased significantly already with 1 mM butyrate. This difference implied the appearance of a "coupling factor" between the receptor and adenylate cyclase. New evidence indicates that the coupling factor may be identical to the "nucleotide binding subunit" (N or G/F) described by others. It is now planned to measure the synthesis and number of these coupling factors in HeLa cells permeabilized with lysolecithin and in a butyrate resistant HeLa mutant. Previous reports had indicated that cholera toxin maximally activates adenylate cyclase activity. This has now been disproved because isoproterenol and cholera toxin together produce a 2.5-fold higher adenylate cyclase activity than either the toxin or isoproterenol alone.

Embryonic quail skeletal myoblasts were used to study the appearance of B-adrenergic receptors during the differentiation to muscle cells. While they proliferate, myoblasts contain fewer than 10 B-adrenergic receptors per cell. During the fusion of myoblasts to form differentiated myotubes, approximately 800 B-adrenergic receptors are formed per diploid nucleus, whereas the specific activity of adenylate cyclase (measured by fluoride stimulation) does not seem to change. The Laboratory will now attempt to determine how the synthesis of these receptors is controlled during differentiation.

Other receptor studies concerned the binding of diazepam to different cell types. Two types of diazepam receptors have been identified: 1) a "central" type, which is found mostly in CNS tissue and is characterized by the ability of clonazepam, but not the benzodiazepine RO5-4864, to displace the bound diazepam; 2) a "peripheral" type, which is found on non-neuronal peripheral tissue such as glioma and from which diazepam can be displaced by RO5-4864 but not by clonazepam. It was also found that certain tissue culture techniques allow the enrichment of rodent embryo neurons. This culture technique is currently used to selectively increase the number of neuronal cells and to identify the intracellular biochemical changes resulting from diazepam binding to these cells. Such studies should make it possible to select neurons (that have not been transformed into cancer cells) and study their response to various signals. Present results suggest that only neurons have diazepam receptors of the central type.

4. Teratogenic examinations in mouse embryo cultures. Earlier experiments in this Laboratory had shown that many lipophilic acids with high lipophilicity (i.e. high partition coefficient) strongly inhibited mammalian cells in culture. Because a number of these compounds were known teratogens, although their mechanism of action was unknown, it was postulated that any lipophilic acid that was strongly inhibitory to mammalian cell cultures would be teratogenic if it could reach the embryo. To examine this contention, rodent embryo cultures, techniques for which have recently been developed, are the ideal tool because problems of absorption, metabolism, excretion, etc., arising in whole rodents can mostly be avoided. Consequently, a mouse culture

was developed from which embryos can be obtained routinely at the ninth day of gestation and can subsequently be grown for 48 hours in rat serum. Without the addition of a drug the embryos develop completely normally. Detailed studies of the anticonvulsant diphenylhydantoin and valproate have shown that these compounds produce various developmental defects including malformations of the brain, spinal cord, craniofacial structures, abnormalities in body curvature, and overall growth retardation. Significantly, these effects were observed within the concentration range found in the plasma of treated patients. It can therefore be expected that these compounds could cause teratogenic effects if they could reach the embryo during organogenesis and expose it to a drug concentration similar to that in the blood. In fact, diphenylhydantoin has been shown by others to produce cleft palate in rodents.

CONTRACT NARRATIVE
Laboratory of Molecular Biology
Fiscal Year 1980

UNIVERSITY OF VIRGINIA (N01-NS-82391)

Title: Large Scale Preparation of VSV and its DI Particles

Contractor's Project Director: Dr. Jay C. Brown

Current Annual Level: \$70,387

Objectives: To establish conditions for the growth and purification of VSV and four of its defective particles which will reproducibly yield materials of the requisite of purity and activity, and to supply such materials to the Laboratory of Molecular Biology, IRP/NINCDS.

Major Findings:

a) Conditions and procedures have been devised for the purification of the virus particles. Materials prepared by this new scheme meet the specifications set forth in the contract.

b) The contractor has delivered to the Laboratory of Molecular Biology, IRP/NINCDS, the amounts of purified VSV and DI particles stipulated in the contract.

Significance to the NINCDS Program and Biomedical Research: The procedures and materials developed under this contract are immediately used by the Molecular Virology Section of our laboratory. This contract, therefore, forms an integral part of that Section's research program, namely, the regulation of viral nucleic acid synthesis in animal cells. This contract has supplied the Program with the raw materials for RNA sequencing of the viral genomes. These studies have characterized sites on the chromosomes that are important for autointerference, DI particle genesis and the replication of the viral genome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02026-08 LMB
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Regulation of Viral Nucleic Acid Synthesis in Animal Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div> PI: R. A. Lazzarini, Head, Molecular Virology Section OTHER: I. Chien Chemist R. Herman Staff Fellow M. Schubert Staff Fellow M. McCarthy IPA J. Condra Staff Fellow F. Yang Visiting Fellow </div> <div style="text-align: right; width: 150px;"> LMB NINCDS LMB NINCDS LMB NINCDS LMB NINCDS LMB NINCDS LMB NINCDS </div> </div>		
COOPERATING UNITS (if any) Department of Neurology, Laboratory of Neurovirology, Johns Hopkins University, School of Medicine; Department of Microbiology, Duke University, School of Medicine		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Virology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md., 20205		
TOTAL MANYEARS: 6.0	PROFESSIONAL: 4	OTHER: 2
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div>		
SUMMARY OF WORK (200 words or less - underline keywords) The long range objective of this project is the description of the component molecular events involved in the replication of the negative strand viruses (myxo, paramyxo, rhabdo, arena and bunya viruses). The topics that are currently being investigated are: <ol style="list-style-type: none"> 1. The origin of DI particles. 2. The mechanism of mRNA synthesis in VSV infected cells. 3. Nucleocapsid assembly. 		

Project Description:

Objectives: Viral diseases of the central nervous system (CNS) usually occur as a complication rather than a normal consequence of infection. Nevertheless, many members of the myxo-, paramyxo-, rhabdovirus family, either exceptionally or a normal consequence, infect the CNS, causing encephalitis or meningitis. Despite their importance to medical neurology, very little is known about the regulation and mode of replication of these viruses in the host organism. From what little is known, it is clear that their replication is very different from that described for polio virus or the RNA tumor viruses. Furthermore, the myxo-, paramyxo-, rhabdovirus infections also are distinguished in that they frequently elaborate defective interfering (DI) particles and exhibit evidence of autointerference and viral persistency. The description of the component molecular events involved in the replication of these viruses, the generation of defective interfering particles, autointerference and viral persistency are the subject of this project. It is anticipated that the study will delineate characteristics that can be exploited in containing and limiting viral infection to non-neural tissues or in the treatment or prevention of the viral infection.

Major Findings: Three major areas of our program have been pursued during the last year, and although interrelated, each area is discussed separately.

1. The origins of defective interfering virus particles. We have studied the structure of the DI chromosomes of several negative strand RNA viruses by RNA sequencing, electron microscopy and molecular hybridization. These studies indicate that there are three general types of DI particles. The first type contains genetic information from both the 5' and 3' portions of the parent genome but lacks some of the central portions. This DI is a true deletion mutant. The second and largest group contains genetic information from the 5' portion of the chromosome and has complementary terminal sequences of 45 to 70 nucleotides long. A third type of DI also contains genetic information from the 5' portion of the chromosome but it is covalently linked to the exact complementary sequence. Our studies of the DI genome structure not only have established the multiplicity of types but have shown that DI particles are formed by aberrant replicative events; that is, they are a type of mutant. Each type of DI is formed by a different, but related, aberration as shown in the diagram below.

In the first, the polymerase copying the template ABC, jumps from one position to another one farther downstream on the same template and resumes synthesis there. The product synthesized by this polymerase contains a deletion corresponding to the portion of the chromosome that was not copied. In the second type of replicative aberration the polymerase also jumps and resumes synthesis, but in this model it jumps to a position on its own daughter strand that is 45 to 70 nucleotides from the end. Continued synthesis at this point creates complementary termini on the daughter strand. In the third, two polymerase molecules copy the same template in tandem. For unknown reasons the first polymerase becomes attenuated in its forward motion, and the second polymerase catches up with it. When its forward motion is

impeded, the second polymerase jumps to the daughter strand of the first polymerase and resumes synthesis there. The product of this type of aberration is a DI genome that is wholly self-complementary. The common feature of these mechanisms is that the polymerase jumps to a new template or position on the old template while keeping its newly synthesized daughter strand in tow and resumes synthesis by adding nucleotides to the appended daughter strand. Thus, the ability to generate DI particles is an intrinsic property of the polymerase. We believe that it is an important characteristic of the VSV polymerase and one which has implication for the synthesis of viral mRNA (see diagram).

2. The mechanism of mRNA synthesis in VSV infected cells. The five species of VSV mRNA are capped at their 5' termini and polyadenylated at their 3' termini. A number of observations have led to the speculation that these mRNAs are not individually synthesized but are derived from a large polycistronic precursor by nucleolytic processing. However, other observations support an alternative explanation—that the RNA polymerase initiates and terminates synthesis at the beginning and end of each mRNA and that the five mRNAs are the result of five separate RNA synthetic events.

During the last year we have continued our studies on the putative polycistronic VSV mRNA that we described two years ago. At that time we speculated on the basis of electron micrographic studies that these unusual RNAs consisted of two mRNAs connected by a long stretch of poly(A). We have now succeeded in determining the exact nucleotide sequence of these RNAs and have proven that they are precisely what they were predicted to be. Not only were these RNAs composed of two mRNAs linked by an intervening stretch of poly(A), the viral polymerase itself must have synthesized the poly(A) by chattering at a tract of seven uridylate residues that lie at the end of each gene (see diagram). This mechanism which we had previously proposed involves an unusual interaction of the polymerase and template. That is, the polymerase must read a sequence (U_7), synthesize the cognate RNA (A_7) but either fail to translocate along the template or translocate but jump back. This property is reminiscent of the aberrations that give rise to DI particles. This work has yielded the first clear identification of a mRNA polyadenylating enzyme in any eukaryotic cell or viral system.

The existence of polycistronic mRNA which contains large tracts of poly(A) suggests that VSV monocistronic mRNA may arise by cleavage and processing of this polycistronic RNA. This possibility is diagrammed at the bottom of the figure. A final decision on the relevance of these linked mRNAs to the mechanism of message synthesis cannot be made yet and may depend upon a future demonstration that the polycistronic transcripts can be processed into mature capped and methylated viral mRNAs as shown in the figure.

3. Viral assembly. Within the infected cell, the chromosome of negative strand RNA viruses exists as a nucleocapsid structure containing single stranded RNA and many subunits of a specific protein, the nucleocapsid protein. These structures serve as a template for the synthesis of genome-length (+) RNA that are the presumed templates for the negative strand RNA nucleocapsids that will eventually mature into progeny virus particles.

This process of genome RNA synthesis has been termed replicative RNA synthesis and has not been unequivocally demonstrated in cell-free systems. Data from a number of laboratories indicates that replicative RNA synthesis in infected cells requires concomitant protein synthesis. Although attempts have been made to couple in vitro RNA transcription with translations systems, the in vitro synthesis of nucleocapsids has not been accomplished, and there has been no easily manipulatable system to study nucleocapsid assembly. In order to circumvent the shortcomings of the in vitro systems and the experimental inflexibility of the intact cell, we have developed a permeable cell system to study negative strand RNA virus nucleocapsid assembly. Permeabilization of the mammalian cells is accomplished with lysolecithin in the cold. Infected cells so treated become permeable to nucleoside triphosphates and a variety of other compounds which do not normally penetrate the cell membrane. After permeabilization, cells incorporate nucleoside triphosphates into RNA at a rate that closely resemble that found in vivo. Furthermore, full length nucleocapsids of both polarities are formed and contain newly synthesized RNA and protein. The great advantage of this method is that it closely approximates conditions found during normal viral infections of intact cells. Since the permeabilization takes place in the middle of the infectious cycle it allows the continued interaction of the virus with host cell factors that may strongly influence the course of the infection and which are not available in a completely in vitro system. The permeable cells system we have developed represents the only in vitro-like system in which unequivocal genome replication and nucleocapsids synthesis has been shown to occur. We anticipate that the achievement of a permeabilized cell system which synthesizes nucleocapsids will enable us to study the assembly process in ways not available otherwise and eventually to understand this complex but central assembly event.

Proposed Course of Project: 1. To further investigate the RNA sequences of the regulatory regions on DI and infectious particle genomes. 2. To obtain specific RNA substrates for the putative viral RNA processing enzymes and to attempt to demonstrate the existence of enzymes capable of specifically cleaving the polycistronic mRNAs, converting them into mature, functional mRNAs. 3. To investigate the biochemical events leading to the assembly of nucleocapsids from the precursor proteins and RNA. Efforts will be made to investigate the regulation and orchestration of this process.

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Keene, J.D., Schubert, M., and Lazzarini, R.A.: Terminal sequence of vesicular stomatitis virus RNA are both complementary and conserved. J. Virol. 32: 167-174, 1979.

Johnson, L.D., Binder, M., and Lazzarini, R.A.: A defective interfering vesicular stomatitis virus particle that directs the synthesis of functional proteins in the absence of helpful virus. Virol. 99: 203-206, 1979.

Baczko, K., and Lazzarini, R.A.: Efficient propagation of measles virus in suspension cultures. J. Virol. 31: 854-855, 1979.

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- Faulkner, G., Dubois-Dalcq, M., Hooghe-Peters, E., McFarland, H.D., and Lazzarini, R.A.: Defective interfering particles modulate VSV infection of disassociated neuron cultures. Cell 17: 979-991, 1979.
- Keene, J.D., Schubert, M., and Lazzarini, R.A.: Intervening sequence between the leader region and the nucleocapsid gene of vesicular stomatitis virus RNA. J. Virol. 33: 789-794, 1980.
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- Reichmann, M.E., Bishop, D.H.L., Brown, F., Crick, J., Holland, J.J., Kang, C.-Y., Lazzarini, R., Moyer, S., Perrault, J., Prevec, L., Pringle, C.R., Wagner, R.R., Youngner, J.S., and Huang, A.S.: Proposal for a uniform nomenclature for defective interfering viruses of vesicular stomatitis virus. J. Virol. 34: 792-794, 1980.
- Faulkner, G.P., and Lazzarini, R.A.: Homologous interference by defective virus particles. In Bishop, D.H.L. (Ed.): Rhabdoviruses II(9). Boca Raton, Florida, CRC Press, Inc., 1980, pp. 163-176.
- Johnson, L.D., and Lazzarini, R.A.: RNA synthesis by defective interfering vesicular stomatitis virus particles. In Bishop, D.H.L. (Ed.): Rhabdoviruses II(10). Boca Raton, Florida, CRC Press, Inc., 1980, pp. 177-187.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01244-16 LMB
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Control Mechanisms and Differentiation		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: E. Freese, Chief, Laboratory of Molecular Biology LMB NINCDS OTHERS: T. Beaman IPA LMB NINCDS D. Boudreaux Staff Fellow LMB NINCDS K. Dhariwal Visiting Fellow LMB NINCDS T. Endo Visiting Fellow LMB NINCDS A. Hitchins Staff Fellow LMB NINCDS J. Lopez Visiting Associate LMB NINCDS K. Ochi Visiting Associate LMB NINCDS N. Vasantha Visiting Associate LMB NINCDS B. Uratani Visiting Fellow LMB NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Developmental Biology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland, 20205		
TOTAL MANYEARS: 11.3	PROFESSIONAL: 9.6	OTHER: 1.7
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Sporulation normally starts when the growth medium runs out of rapidly metabolizable carbon or nitrogen sources, phosphate or amino acids. The ultimate metabolite controlling this differentiation process was shown to GTP, whose partial deficiency initiates sporulation. Partial amino acid deficiency also causes sporulation because it invokes the stringent response, including the synthesis of ppGpp, and thereby causes a decrease of GTP. The phenomenon of sporulation initiation was analyzed under conditions under which normal sporulation was prevented by the presence of excess glucose, ammonium ions and phosphate. In this medium, several inhibitors of purine nucleotide synthesis and purine analogs induced sporulation, whereas inhibitors of other biochemical reactions were ineffective. Four phenomena, catabolite repression of enzyme synthesis, manganese requirement of sporulation,		

transport changes and DNA transformation, normally correlated with sporulation could be dissociated from it. GTP deprivation causes a preferential reduction in ribosomal RNA synthesis and the onset of extensive protein turnover. It also causes the increase of citric cycle enzymes, the mechanism of which was determined. The properties of a mutant deficient in the branched fatty acid synthesis were examined.

Project Description:

Objectives: The differentiation of microorganisms and of certain cell types in higher organisms begins when rapidly metabolizable carbon or nitrogen sources or phosphate have been exhausted, or when the cells are exposed to a sudden shiftdown from an amino acid containing medium to one lacking most amino acids. Recent results in the laboratory have established that GTP decreases under all these conditions and have furthermore demonstrated that the decrease of GTP is sufficient to initiate sporulation. Consequently, the role of GTP in the initiation of sporulation and the consequences of a GTP decrease for other cellular reactions have been examined. As many cellular changes are normally correlated with sporulation, we wanted to know which of them are actually necessary for it. Since the stringent response to amino acid starvation has a drastic effect on many cellular reactions, we also examined whether this effect could cause sporulation and whether it was necessary for it.

Methods Employed: *Bacillus subtilis* was grown in a number of different media in which sporulation could normally be observed and the change of nucleotide pools was measured by ^{32}P incorporation and thin layer chromatography and high pressure chromatography of the formic acid extracts. A relaxed (relA) mutation was introduced into different strains by transformation to allow a decision as to whether the stringent response was necessary for sporulation in certain media. To obtain partial deprivation of amino acids, mutants were isolated that were deficient in the transport of different amino acids and also auxotroph for these amino acids. To determine the concentration of CoA-derivatives a thin layer chromatographic technique was developed whereby most sulfur-containing compounds could be separated.

Major Findings: 1. Initiation of sporulation by normal nutrient deprivation. When we determined the changes of nucleotides under conditions under which sporulation was initiated by nutrient deprivation, we found that GTP decreased in all cases, whereas the other nucleotides increased in some and decreased in other cases. When sporulation was initiated by the specific deprivation of nitrogen or phosphate, all nucleotides decreased, which is not surprising. The deprivation of carbon compounds had a more mixed effect which depended on the amino acids presence in the original growth medium. If cells were transferred from a glucose to a lactate medium in which they can grow only extremely slowly, the concentration of P-ribosyl-PP decreased and consequently all derived nucleotides decreased. However, the decrease of GTP was much more pronounced than that of ATP, while ppGpp increased. If cells were transferred from a medium containing most amino acids to one containing only a slowly metabolizable carbon source, the stringent response to amino acid deprivation set in so rapidly that the cell did not have time to use up

P-ribosyl-PP, which increased. This increase of ppGpp caused a corresponding decrease of GTP. Other experiments, using granaticin as inhibitor of leucyl-tRNA activation in the presence and absence of decoynine, demonstrated that the stringent response (ppGpp) specifically inhibits the function of IMP dehydrogenase. If the same experiments were repeated with a relaxed mutant, the ppGpp increase was avoided and sporulation occurred only in those cases in which a general purine starvation had occurred. The results demonstrated that normal sporulation conditions can initiate sporulation via variety of metabolic reactions, all of which lead to the decrease of GTP.

2. Sporulation initiation by partial amino acid deprivation. Since sporulation induction was observed only when purine synthesis was partially but not completely inhibited, it appeared possible that partial amino acid starvation might also initiate sporulation, especially if it would be done in a stringent strain leading to a partial stringent response. To examine this possibility, mutants were isolated that were on the one hand auxotroph for a particular amino acid and on the other hand unable to actively transport the amino acid or its precursor into the cell so that the growth of the mutant would depend on the concentration of the amino acid (or precursor) in the medium. This was successfully done for aspartic, isoleucine-valine, and methionine requiring mutants. To determine the importance of the stringent response, a relaxed (relA) mutation was also introduced into the strains in an isogenic fashion. The results have demonstrated that partial deprivation of amino acid supply to the cell invokes a partial stringent response, including the synthesis of ppGpp and pppGpp, and initiates extensive sporulation. However, this phenomenon is completely abolished in the relaxed strains. The increase of ppGpp is correlated with a rapid decrease of GTP in the stringent and a slow, less extensive decrease in the relaxed strain. The relaxed strains can nevertheless be induced to sporulate, even during partial amino acid deprivation, by the addition of decoynine or by guanine deprivation in a guanine auxotroph, both conditions that reduce the concentration of GTP further. The results have demonstrated that conditions of stringent control do initiate sporulation but they are not necessary for the initiation which can also be obtained by any other condition leading to a sufficient decrease of GTP.

3. Initiation of sporulation by purine nucleoside analogs and utilization of this phenomenon to determine the correlation between sporulation and other physiological effects. As decoynine is an adenosine analog which inhibits GMP synthetase, several other adenosine analogs were tested for their effect on guanine nucleotide synthesis and sporulation. Cordycepin, formycin and psicofuranine were found effective. In addition, the inhibitors of IMP dehydrogenase, virazole and mycophenolic acid, reduced GMP synthesis and induced sporulation. Also several cytokinins were effective.

The most efficient sporulation inducer is decoynine; it was used to examine several correlations.

a.) Sporulation normally requires manganese as absolutely necessary metal. Several enzymes use manganese as co-factor and therefore were implicated as possibly being required for sporulation. We have shown that

decoyinine can induce sporulation in the absence of manganese.

b.) Glucose normally suppresses sporulation and represses the synthesis of inducible enzymes (catabolite repression). We demonstrated that these two phenomena are not strictly correlated because decoyinine can induce sporulation in the presence of glucose but it does not relieve catabolite repression of enzyme synthesis.

c.) Conditions of nutrient deprivation which normally lead to sporulation also change the cells so that they become sensitive to transformation by DNA. Since early blocked sporulation mutants also lost their ability to be transformed, it had been assumed that some early changes required for sporulation made cells transformable. However, under conditions of sporulation induction by decoyinine or guanine deprivation of a guanine mutant, transformation is not enabled. Therefore, conditions leading to sporulation do not suffice to enable transformation.

d.) During sporulation at the end of growth in a nutrient medium many transport mechanisms are inactivated. This was thought to be necessary for sporulation, e.g., to protect the differentiating cells from media influences. However, after sporulation induction by decoyinine, the transport mechanisms are maintained.

4. Consequences of GTP deficiency for RNA in protein synthesis. The decrease of GTP resulting in the initiation of sporulation has a number of consequences which have been investigated. The synthesis of ribosomal RNA proteins is rapidly turned off, whereas messenger RNA and specific enzymes or other proteins can still be produced. This control of ribosomal synthesis, which is usually associated with the stringent response mechanism but which is in our case observed without any increase of ppGpp, is under investigation. Of the proteins normally made during sporulation, some showed very little increase, apparently due to the glucose present under our sporulation conditions, while others increased almost as much as usually observed. The latter include a proteolytic activity leading to much protein turnover and glucose dehydrogenase which is made only inside the forespores. These enzymes apparently are not controlled by catabolite repression, yet are under the control of GTP.

The GTP deprivation also causes an increase in citrate synthase, aconitase and alpha-ketoglutarate dehydrogenase. Alpha-ketoglutarate dehydrogenase can also be induced by acetate, and its specific activity is elevated in mutants having high intracellular acetyl-CoA concentrations. Acetyl-CoA also increases during GTP deprivation apparently because fatty acid synthesis is reduced; this explains the induction of alpha-ketoglutarate dehydrogenase. The increase of this enzyme in turn causes a decrease of alpha-ketoglutarate, which represses citrate synthase and aconitase. The investigation of the GTP effect on sporulation has thus indirectly led to an explanation of the control of certain citric acid cycle enzymes.

5. Properties of a mutant deficient in branched chain fatty acid synthesis. B. subtilis incorporates branched chain fatty acids into the

phospholipids in the membrane, apparently in order to maintain a high enough membrane fluidity and perhaps for more specific purposes. In order to determine the requirement for branched chain fatty acids for sporulation, we have analyzed a mutant that requires branched chain fatty acids for growth. We found that any branched chain or otherwise kinky fatty acid allows normal growth and sporulation of the mutant. An investigation of the mutation showed that it produces an acetyl-CoA:ACP transacylase with lower affinity for branched acyl-CoA substrates. The mutation maps in the *acs/hisB* region. Strains containing this mutation require higher than normal intracellular concentrations of branched acyl-CoA precursors to enable the synthesis of branched chain fatty acids at rates sufficient for normal growth. Growth of the mutant enabled by valine and isoleucine is inhibited by butyrate and other straight chain fatty acids at concentrations (0.1mM) which do not inhibit growth of the standard strain; the inhibition is prevented by short branched fatty acids. The results suggest that the acyl-CoA:ACP transacylase activity of *B. subtilis* either consists of one enzyme with separate sites for the acyl-CoA precursors of branched and straight chain fatty acids or is composed of two enzymes.

Proposed Course of Project. Several of the investigations will be continued. For example, we want to understand which protein synthetic mechanisms are under the control of GTP, which under that of ppGpp and which are under the direct control of glucose repression. To separate the effect of the ppGpp increase from that of the GTP decrease we want to isolate mutants in which the two phenomena are uncoupled. We also want to isolate mutants in which sporulation is no longer under the control of GTP and determine their biological control mechanism. This should enable us to determine the protein component on which GTP acts in order to control sporulation. Our observation that normal nutritional conditions can lead to sporulation, either via a deprivation of purines or via a GTP decrease caused by the stringent response, makes it now possible to check which inhibitors or mutations interfering with sporulation in one medium are effective only under some sporulation conditions and which interfere with the sporulation process under all conditions. We will also determine the mechanism whereby GTP deprivation causes an immediate cessation of ribosomal synthesis.

Publications:

Freese, E., Heinze, J.E., and Galliers, E.M.: Partial purine deprivation causes sporulation of *Bacillus subtilis* in the presence of excess ammonia, glucose and phosphate. J. Gen. Microbiol. 115: 193-205, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01886-10 LMB															
PERIOD COVERED October 1, 1979 through September 30, 1980																	
TITLE OF PROJECT (80 characters or less) Developmental Cytology																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: E. B. Freese</td> <td style="width: 30%;">Biologist</td> <td style="width: 30%;">LMB NINCDS</td> </tr> <tr> <td colspan="3">OTHERS: M. Chu</td> </tr> <tr> <td></td> <td>Visiting Fellow</td> <td>LMB NINCDS</td> </tr> <tr> <td>A. Hartig</td> <td>Visiting Fellow</td> <td>LMB NINCDS</td> </tr> <tr> <td>J. Kandala</td> <td>Visiting Fellow</td> <td>LMB NINCDS</td> </tr> </table>			PI: E. B. Freese	Biologist	LMB NINCDS	OTHERS: M. Chu				Visiting Fellow	LMB NINCDS	A. Hartig	Visiting Fellow	LMB NINCDS	J. Kandala	Visiting Fellow	LMB NINCDS
PI: E. B. Freese	Biologist	LMB NINCDS															
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A. Hartig	Visiting Fellow	LMB NINCDS															
J. Kandala	Visiting Fellow	LMB NINCDS															
COOPERATING UNITS (if any) None																	
LAB/BRANCH Laboratory of Molecular Biology																	
SECTION Developmental Biology Section																	
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md., 20205																	
TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.7	OTHER: 0.3															
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINCRS <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINCRS <input type="checkbox"/> (a2) INTERVIEWS											
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<input type="checkbox"/> (a1) MINCRS <input type="checkbox"/> (a2) INTERVIEWS																	
SUMMARY OF WORK (200 words or less - underline keywords) <p>In <u>Bacillus subtilis</u> the initiation of sporulation by partial deprivation of amino acids was examined. Stringent cells, showing the stringent response to amino acid deprivation; i.e., accumulating ppGpp, sporulated during partial amino acid (methionine) deprivation, whereas the relaxed strain did not. However, this was due to the associated GTP decrease because the relaxed strain could also sporulate upon addition of decoyinine, an inhibitor of GMP synthetase.</p> <p>In the yeast <u>Saccharomyces cerevisiae</u>, metabolic changes and sporulation were measured during conditions of nitrogen and carbon limitation. It was also determined why yeast cells grown in glucose can subsequently not grow on acetate, although they can grow on ethanol.</p>																	

Project Description:

Objectives: Various experiments in this laboratory had shown that partial deprivation of guanine nucleotide synthesis leads to sporulation of *Bacillus subtilis*. To determine whether amino acid deprivation could also have this effect, it was necessary to develop a technique with which an amino acid could be supplied to the inside of the cell at a slow but constant rate. We also wanted to know whether any sporulation observed was due to the stringent response to amino acid starvation. Therefore we incorporate it into the mutants used also the relaxed mutation which prevents the stringent response.

To examine whether differentiation is similarly controlled in eukaryotes, we used the yeast *Saccharomyces cerevisiae*. Its cells have to undergo meiosis before they can sporulate; the mechanism of meiosis is not understood. First of all we wanted to establish conditions under which *S. cerevisiae* could sporulate not only under the usually employed conditions of nitrogen limitation but also by carbon limitation in the presence of nitrogen. This would allow us to manipulate the metabolic machinery to determine which compound controls differentiation. We encountered a number of problems: The adaptation of yeast to new metabolic conditions is much slower than that of bacteria and in certain media essentially impossible; this phenomenon was therefore fully investigated. We also found that the rate of transport of the carbon compounds was critical for the control of sporulation and investigated the mechanisms involved.

Methods Employed: A methionine-requiring mutant was also made relaxed (relA) as detected by slow growth on certain inhibitory amino acid analogs and directly verified by continued RNA synthesis after amino acid deprivation. Sporulation was measured 10 hr after transfer of the *B. subtilis* mutant to a synthetic medium containing different amounts of D-methionine whose concentration controls the rate of growth of the strains used. During yeast growth, glucose, pyruvate, acetate and ethanol were monitored by enzymatic methods, ATP was measured by the luciferase assay, and CoA derivatives were measured by high pressure chromatography and thin layer chromatography.

Major Findings: 1. Sporulation of *B. subtilis* during partial methionine deprivation. Since amino acids are actively transported into the cells, their supply to the cell can normally not be controlled by the extracellular concentration (except under conditions under which the compound is almost immediately used up). We have found that an L-methionine requiring mutant can grow at different rates if it is supplied with different concentrations of D-methionine instead of the L-methionine. As the methionine racemase is quite active, the transport of D-methionine apparently is rate limiting. When cells grown on L-methionine were transferred to media containing different concentrations of D-methionine, they sporulated excellently (after 10 hr) at an intermediate D-methionine concentration. However, a methionine auxotroph which was relaxed (relA) in its response to amino acid deprivation did not sporulate at any D-methionine concentrations, although its growth rate also depended on the D-methionine concentration. Nucleotide analysis demonstrated that ppGpp and pppGpp increased in the stringent strain immediately after

transfer to the growth rate-limiting D-methionine while GTP rapidly decreased. Both of these changes were greatly reduced in the relaxed strain, which did not sporulate. However, the relaxed strain could also sporulate when its GTP concentration was further decreased by the addition of decoyinine or by guanine deprivation of a corresponding guanine auxotroph. Thus, conditions of stringent response initiate sporulation but any other conditions which produce a decrease of GTP can do the same.

2. Adaptation of *Saccharomyces cerevisiae* to gluconeogenic growth conditions. When *Saccharomyces cerevisiae* is grown on a synthetic medium containing glucose and transferred after thorough washing to a medium containing either acetate or ethanol as sole carbon source, cells adapt within 15 hr to grow on the ethanol but they do not adapt for 7 days to grow on acetate. This was surprising because ethanol has to be metabolized via alcohol dehydrogenase and aldehyde dehydrogenase to acetate before it can be used as carbon source. This difference obviously cannot be explained by the findings that gluconeogenic enzymes and the enzymes of the glyoxylate bypath are repressed during growth on glucose have to be synthesized to enable growth at the maximal rate. We have demonstrated that after transfer to acetate as sole carbon source, ATP rapidly decreases to undetectable levels and oxygen consumption completely stops; in contrast, in the presence of ethanol (with and without acetate) ATP and oxygen consumption are maintained at a detectable level during the lag period before growth resumes. This ethanol effect is apparently due to the continued reduction of NAD to NADH via the alcohol and aldehyde dehydrogenases, both of which are active in glucose grown cells. The NADH in turn can be re-oxdized via the electron transport system and thereby regenerate ATP. The understanding of the gluconeogenic adaptation explains why glucose grown cells sporulate so badly on acetate and makes it now possible to design media for faster adaptation. It is likely that these control mechanisms operate in all eukaryotic organisms.

3. Sporulation of yeast under conditions of carbon deprivation. Yeast sporulation is usually measured after the transfer of cells from a growth medium to potassium acetate. The acetate serves as carbon source while the medium contains no nitrogen or phosphate sources. Some yeast strains can then sporulate within 20 hrs, apparently due to nitrogen limitation because addition of ammonia prevents the sporulation process (whereas addition of phosphate has no effect). To examine the mechanism controlling the initiation of meiosis and sporulation, we wanted to determine whether sporulation could also be initiated by carbon deprivation; this would indicate that the mechanism of control is similar to that observed in *Bacillus subtilis*. We found that it was very difficult to maintain a constant pH of the yeast medium because the usually used gluconeogenic carbon sources, acetate and pyruvate, were added as potassium salts and during their metabolism were largely converted to CO₂, thereby causing a pH increase in the medium. As the uptake rate of these compounds was in turn controlled by the pH in the medium, it was difficult to maintain constant rates of uptake and thus establish different rates of growth. This was solved partially by the use of high buffer concentrations but more adequately by the employment of a pH-stat. We found that at certain concentrations of pyruvate and at certain elevated pH values the rate of growth was slow and good sporulation was observed within 20 hr

after transfer to this medium. The effect depended on the precise pH which determined the concentration of neutral pyruvate molecules, apparently the species that enters the cell.

Proposed Course of Project: As it is now apparent that yeast sporulation is controlled by a nitrogen and carbon containing compound, it is likely that the control is similar to that observed in *B. subtilis*. We want to use the lead provided by *B. subtilis* studies to determine which compound is used as a signal for the start of meiosis and differentiation. To this end we will study different media conditions, determine the change of nucleotides and other compounds, and correlate these changes with sporulation. To determine a causal relationship, we will use or isolate mutants in different major biochemical pathways, including those of nucleotides and amino acids, and determine partial limitation of which compound will lead to sporulation.

Publications:

Vasantha, N., and Freese, E.: The role of manganese in growth and sporulation of *Bacillus subtilis*. J. Gen. Microbiol. 112: 329-336, 1979.

Freese, E., Lopez, J.M., and Freese, E.B.: Initiation of bacterial and yeast sporulation by partial deprivation of guanine nucleotides. In Richter (Ed.): Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators. New York, Academic Press, 1979, pp. 127-143.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02365-02 LMB
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Intercellular Communication and Transmembrane Signals		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Richard C. Henneberry, Senior Scientist LMB NINCDS		
COOPERATING UNITS (if any) (1) Biological Psychiatry Branch, NIMH (2) Developmental and Metabolic Neurology Branch, NINCDS		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Developmental Biology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md., 20205		
TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1
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SUMMARY OF WORK (200 words or less - underline keywords) This project concerns the mechanisms by which extracellular signals are received by cells, with emphasis on the biochemical events involved in the transmission of such signals across membranes. During this reporting period, we have continued to study the interaction of hormones/neurotransmitters with their receptors in mammalian cells in culture. The inducible B-adrenergic receptors in HeLa cells, which we previously described, were used to study the components of the receptor-adenylate cyclase system. In addition, the phenomenon of agonist-induced desensitization was examined, and evidence presented for a functional role for phospholipids in the domain of the receptor. Finally, new methods which permit the cultivation of rodent embryo neurons have been adapted; CNS-type receptors for diazepam (valium) were demonstrated on these cells, and new results on the nature of these receptors were reported.		

Project Description:

Objectives: The major objective of this project is to elucidate the molecular mechanisms underlying the receipt of biochemical signals by individual cells, with particular emphasis on a) the means by which hormone/neurotransmitter messages are transmitted across the plasma membrane; and b) the regulation of receptor synthesis and degradation. Cultured mammalian cells will be used when possible. We will continue to study the inducible B-adrenergic receptors which we previously described in HeLa cells, and attempt to better understand the role of synthesis and breakdown of phospholipids in receptor function, the relationship between phospholipid metabolism and desensitization, and the role of the various components of the receptor-adenylate cyclase system. Our analysis of receptor function will be extended to other cell types using both established cell lines and primary cultures.

Methods Employed: Established tissue culture cell lines were cultivated by standard methods. Primary cultures of embryonic rat cortex or whole brain were established from rats in the 14th day of gestation by modifications of methodology recently published by several other laboratories. Embryonic quail myoblasts were isolated from the pectoral muscle of 10-day-old quail eggs by established procedures. The various hormone/neurotransmitter receptors were quantified by binding assays using the appropriate radioactive ligand. Cyclic AMP was measured by a standard protein kinase binding procedure.

Major Findings:

1. We have previously described the inducible B-adrenergic receptors in HeLa cells. The number of receptors per cell, but not the cellular content of adenylate cyclase, increases markedly with exposure of cells to butyrate. This system provides a very useful model for studying receptor-adenylate cyclase interactions since the B-adrenergic receptors can be induced in two forms, either "coupled" to or "uncoupled" from adenylate cyclase; coupling of receptor and enzyme can be effected by altering the concentration of butyrate in the culture medium. We have also previously described the inducibility of cholera toxin receptors by butyrate in the same HeLa cell line. This year we have used these cells for further studies on the *in vivo* role of GTP in the activation of adenylate cyclase by B-hydroxylated catecholamines. Previous reports from other laboratories have indicated that cholera toxin maximally activates a cell's adenylate cyclase. Therefore, in HeLa cells fully induced for cholera toxin receptors, isoproterenol would not be expected to activate adenylate cyclase in cells exposed to the toxin. However, we have found a synergistic effect between the two agents. We have also obtained evidence that the "coupling factor" we previously proposed in HeLa cells may be identical to the nucleotide binding subunit (N or G/F) described by others. Preliminary results suggest that butyrate may induce coupling by increasing the rate of synthesis of the N subunit. We are now trying to measure N-subunits directly by NAD-dependent ribosylation of N in cells exposed to cholera toxin. We have begun to experiment with lysolecithin-mediated permeabilization of HeLa cells; such cells would permit us to directly manipulate the levels of nucleotides available to the receptor-adenylate cyclase complex. Additional results concerning the components of this complex

have been derived from further studies on the butyrate resistant HeLa cell mutant, BR-2, which we have previously described. We selected this mutant for its ability to grow normally in the presence of butyrate and later found that the mutant is unable to metabolize butyrate. Surprisingly, B-adrenergic receptors are induced by butyrate in these cells; experiments to determine if the BR-2 receptors are coupled to adenylate cyclase are in progress. In addition to aiding in the analysis of the components of the receptor-adenylate cyclase complex, the results obtained with BR-2 should contribute to our understanding of butyrate's mechanism of action. We have also studied the phenomenon of agonist-induced desensitization in cultured cells; as in whole animals, sensitivity is lost when receptors are chronically occupied by agonists. Inhibitors of phospholipase A₂ block desensitization, whereas activators of the same enzyme cause a decrease in hormone sensitivity; both results support the functional role for phospholipids in the domain of the receptor which we previously proposed.

2. We have used embryonic quail skeletal muscle cells to study the development of hormonal responsiveness in differentiating cells. These myoblasts fuse and differentiate in a synchronous manner, going from 0 to 70 percent fusion within 24 hrs. Proliferating myoblasts contain fewer than 10 B-adrenergic receptors per cell, but receptors begin to appear in parallel with the fusion process. The adenylate cyclase of the fusing myoblasts becomes sensitive to activation by isoproterenol over the same time course. Well-differentiated myotubes contain approximately 800 B-adrenergic receptors per diploid nucleus. The increase in receptor number appears to reflect de novo synthesis. However, the amount of adenylate cyclase activity as measured by fluoride stimulation does not change during fusion. Apparently, newly synthesized receptors couple with pre-existing adenylate cyclase molecules. These results are consistent with the physiological effects of B-hydroxylated catecholamines in mature muscle.

3. From pharmacological evidence it is clear that receptors for diazepam and related compounds occur both in the CNS and in peripheral tissues; these two receptor types can be distinguished by the ability of different compounds to displace diazepam. Our earlier studies on diazepam receptors in the C₆ astrocytoma cell line showed that phospholipid methylation was stimulated when receptor was occupied by agonist. However, we also found that the diazepam receptors on C₆ cells are of the peripheral type rather than the central type; the latter are of major interest with respect to the mode of action of these drugs. For this reason we adapted methodology recently described from other laboratories which permits enrichment for and extended maintenance of rodent embryo neurons in culture. Diazepam binding was pharmacologically characterized by its displacement by the centrally active benzodiazepine, clonazepam, but not by the centrally inactive benzodiazepine, RO5-4864; thus, these receptors are of the central type. In contrast, membranes obtained from peripheral tissue and non-neuronal cultures of glioma and neuroblastoma cells contain peripheral-type diazepam receptors. In neuronal cultures established from rat embryos at day 14 in gestation, diazepam binding increased with the age of the cells in culture; an increase of more than 2-fold was shown from day 5 to day 11 indicating the continuation of neuronal differentiation in these cells. We are currently studying the biochemical responses to receptor

occupancy in this system. In preliminary experiments we have also shown central-type diazepam receptors in embryonic rat neurons cultured by an alternative method, aggregate culture; results to date suggest that this method may permit more extensive neuronal differentiation.

Proposed Course of Project: We will continue to study the receptor-adenylate cyclase complex in cultured cell models. Efforts to extend our studies into the rapidly emerging area of intracellular communication involving the neurologically important small peptide hormones will continue. Model systems based on easily cultivated cell lines will play a major role in this project. However, neuronal culture will receive increasing emphasis. This approach appears to have great potential for permitting direct study of neurologically significant problems. In both the primary culture of neurons and the long-term culture of established cell lines special emphasis will be placed on the development of chemically defined, serum-free growth media. Such media will permit a direct approach to the interactions among the various hormones to which a single cell responds.

Publications:

Strittmatter, W.J., Hirata, F., Axelrod, J., Mallorga, P., Tallman, J.F., and Henneberry, R.C.: Benzodiazepine and B-adrenergic receptor ligands independently stimulate phospholipid methylation. Nature 282: 857-859, 1979.

Mallorga, P., Tallman, J.F., Henneberry, R.C., Hirata, F., Strittmatter, W.T., and Axelrod, J.: Mepacrine blocks B-adrenergic agonist-induced desensitization in astrocytoma cells. Proc. Natl. Acad. Sci. 77: 1341-1345, 1980.

Parent, B., Tallman, J.F., Henneberry, R.C., and Fishman, P.H.: Appearance of B-adrenergic receptors and catecholamine-responsive adenylate cyclase activity during fusion of avian embryonic muscle cells. J. Biol. Chem. In press.

Janes, G.: The effects of variations in intracellular GTP on the B-adrenergic-adenylate cyclase system in HeLa. Dissertation. Georgetown University, 1980.

Hirata, F., Tallman, J.F., Henneberry, R.C., Mallorga, P., Strittmatter, W.J., and Axelrod, J.: Regulation of B-adrenergic receptors by phospholipid methylation. In Pepeu, G., Kuhar, M.J., and Enna, S.J. (Eds.): Receptors for Neurotransmitters and Peptide Hormones. New York, Raven Press, 1980, pp. 91-97.

Tallman, J.F., Henneberry, R.C., Hirata, F., and Axelrod, J.: Control of B-adrenergic receptors in HeLa cells. In Usdin, E. (Ed): Catecholamines: Basic and Clinical Frontiers. Oxford, England, Pergamon Press, 1979, pp. 489-491.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02364-02 LMB
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Development and Teratology in Rodent Embryo Culture		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex; justify-content: space-between;"> <div> PI: E. Freese, Chief, Laboratory of Molecular Biology R. C. Henneberry, Senior Scientist OTHER: A. Bruckner, NIH Expert </div> <div style="text-align: right;"> LMB NINCDS LMB NINCDS LMB NINCDS </div> </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Developmental Biology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md., 20205		
TOTAL MANYEARS: 1.9	PROFESSIONAL: 1.4	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES </div> <div> <input checked="" type="checkbox"/> (c) NEITHER </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) The goal of this project is to determine whether compounds highly inhibitory to mammalian cells in culture are potentially teratogenic. To avoid complications of absorption, metabolism and excretion in whole animals, the recently established system of rodent embryo culture is used. During the initial year of this project, a mouse-breeding colony has been established to provide, on a regular basis, pregnant mice at a particular stage of gestation. Early somite-stage embryos were explanted at day 9 of gestation and cultivated <u>in vitro</u> for up to 48 hrs. Selected lipophilic drugs, in particular the anticonvulsants diphenylhydantoin and valproate, were added to the culture medium; their teratogenicity was assessed by anatomical and histological examinations. Developmental defects, including various neurologic and craniofacial malformations, abnormalities in body curvature, and overall		

growth retardation were observed when the dose of the anticonvulsant drugs was in the range of concentrations found in the plasma of treated patients.

Project Description:

Objectives: The previously inaccessible postimplantation rodent embryo has become available for direct experimentation as a result of improved methodology introduced in recent years. Rodent embryos can now be explanted at the 2-4 somite stage and cultivated for up to 48 hrs. This is the most active period of organ development in the rodent. During this time in culture the embryo appears to develop normally in every respect.

The major objective of this project is to determine whether compounds that are highly inhibitory to mammalian cell cultures merely inhibit the development of rodent embryos in culture or whether they produce teratogenic defects. The compounds will be chosen from a list of highly lipophilic substances, in particular lipophilic acids, which are used as drugs and food additives, and which have been identified by previous studies in this laboratory as potent inhibitors of mammalian cell growth.

The second objective is to use embryo cultures for basic biochemical studies in developmental biology. The time and sequence at which biosynthetic enzymes and cell surface receptors for a variety of hormones and neurotransmitters appear during development will be measured by radioactive or fluorescent probes.

Methods Employed: A breeding colony of ICR mice was maintained in a 12 hr light-dark cycle and bred on a carefully designed schedule to provide mice containing embryos at the 9th day of gestation. Embryos were dissected free of placental material and Reichert's membrane and cultivated in individual vessels in a medium consisting of 90 percent rat serum and 10 percent Tyrode's solution. Compounds to be tested were added directly to the culture medium and teratogenic effects were assessed by anatomical and histological examinations. Antibiotics were not included in the culture medium to avoid possible complications in interpretation of results. A gas flow meter was adapted for monitoring rate of gassing of embryos, and procedures for measurement of such serum components as glucose, lactate, and fatty acids before and after cultures were introduced. To permit the measurement of DNA and protein in single embryos, extrasensitive assay methods were developed.

Major Findings:

1. Early somite-stage embryos at the 9th day of gestation can be readily explanted from pregnant ICR mice and their development can be continued in rat serum. The breeding program must be very carefully designed and monitored to provide reliable embryos of uniform age. When cultivated by methods recently developed in other laboratories, embryos appear to develop normally in every observable respect for 48 hr after explantation.

2. Teratogenic effects were assessed by microscopic examination of intact embryos and of fixed and stained cross sections. Protein and DNA

content were measured by highly sensitive fluorometric procedures to permit estimation in a single 2-somite embryo. The frequency of abnormal development was very low in embryos cultivated by these methods in the absence of test compounds.

3. To evaluate this system for the identification of potential teratogens, several suspected substances were tested. In particular, the anticonvulsant drugs valproic acid and diphenylhydantoin caused a dose dependent increase in the frequency of several developmental abnormalities, including malformations of brain, spinal cord, and craniofacial structures, abnormalities in body curvatures, and overall growth retardation. These effects were observed within the concentration range found in the plasma of treated patients, and are in agreement with the results of whole-animal studies reported by others.

Proposed Course of Project: Additional suspected substances will be tested for teratogenicity and the usefulness and limitations of embryo culture as a method for the screening of potential teratogens will be determined. The results will be correlated with animal test studies and clinical findings. In addition, the development of certain receptors for hormones and neurotransmitters and the influence of certain hormones on developmental changes will be examined.

Publications:

Freese, E., Levin, B.C., Pearce, R., Sreevalsan, T., Kaufman, J.J., Koski, W.S., and Semo, N.M.: Correlation between the growth inhibitory effects, partition coefficients and teratogenic effects of lipophilic acids. Teratology 20(3): 413-439, 1979.

Freese, E.: Mechanism of growth inhibition by lipophilic acids. In Kabara, J. (Ed.): Pharmacological Effect of Lipids. American Oil Chemists' Society, Champaign Illinois, 1978, pp. 123-131.

ANNUAL REPORT

October 1, 1979 through September 30, 1980

Laboratory of Neuro-otolaryngology
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980
Laboratory of Neuro-otolaryngology, IRP
National Institute of Neurological and
Communicative Disorders and Stroke

Jürgen Fex, M.D., Ph.D., Chief

The Laboratory has continued its multidisciplinary approach with the focus on the inner ear and cochlear nucleus of mammalian species, of normal animals as well as of genetically deaf animals. The two Projects of the Laboratory have been advanced, these being Project Number Z01 NS 02216 05 LNO, Inner Ear Neuronal Mechanisms: A Multidisciplinary Analysis, respectively Project Number Z01 NS 02217 05 LNO, Synaptic Transmission and Neuronal Connections of the Mammalian Cochlear Nucleus. In particular, during this fiscal year, the Laboratory contributed with the following new knowledge.

As described in the annual reports of the two preceding years, the high-resolution method for two-dimensional separation of proteins has been worked out in the Laboratory for application to inner ear structures. The previously described study of protein patterns of the stria vascularis, comparing the normal and the genetically deaf waltzing guinea pig, is in press. It has also been found that proteins of the organ of Corti can be analyzed with this technique. The autoradiographic study described in last year's report of in vitro uptake of putative neurotransmitters into the organ of Corti has been published.

Immunocytochemical and microdissection techniques have been worked out in the Laboratory for the study of the distribution of small peptides and proteins in the cochlea of mammals. It has been found that enkephalin-like immunoreactivity is present in efferent neurons in the cochlea. A manuscript of the results is under preparation. As a result of these interesting new findings further studies related to the possible presence of enkephalin in the cochlea are in the planning.

The effects of excitatory amino acid antagonists on sound evoked activity in the auditory nerve have been studied. It was found that DL- α -amino adipate, a dicarboxylic amino acid antagonist, locally applied to the cat cochlea at concentrations of 1 mM and 10 mM, has no effect on the amplitude, latency or shape of click evoked auditory nerve compound action potentials. Thus, the cochlear auditory nerve synaptic receptors are unlikely to be of the aspartate-preferring type. The study has been accepted for publication.

The last few years' studies on glutamate and aspartate as putative transmitters of the auditory nerve in the cochlear nucleus have been extended as follows:

The previously described study on the effects of DL- α -amino adipate on synaptically and chemically evoked excitation of anteroventral cochlear nucleus neurons of the cat has been published. This study has been complemented with a study, also published, using an extended spectrum of sound

for excitation and additional antagonists. Both studies used microelectrode techniques with iontophoretic application of drugs. It was found that auditory nerve evoked activity of single units in the cochlear nucleus was blocked by DL- α -aminoadipate, D- α -aminoadipate, DL- α -aminosuberate, HA966 and magnesium ions, while glutamate diethyl ester rarely affected such activity. The data offer substantial evidence that aspartate is more likely to be the transmitter of the auditory nerve than glutamate.

The biochemical study mentioned last year of enzymes related to the metabolism of aspartate and glutamate has been published. The study showed that glutaminase and aspartate aminotransferase decrease in the cochlear nucleus after lesion of the auditory nerve, indicating that these two enzymes are concentrated in axons and terminals of the auditory nerve. Because of these results, efforts have this last year been directed at localizing aspartate aminotransferase in the cochlear nucleus with immunocytochemistry. The results, now brought together in a manuscript for publication, confirm and extend the biochemical studies indicating that this enzyme is concentrated in primary auditory neurons.

The biochemical, morphological and pharmacological-physiological studies carried out in this laboratory make a stronger case for glutamate and aspartate as transmitter candidates at auditory nerve synapses in the cochlear nucleus than has been made for these substances at any other vertebrate synapses.

The biochemical study mentioned last year of axonal transport in the auditory nerve has been published. This is the first characterization of proteins in the auditory nerve. A presynaptic membrane-associated glycoprotein with a very short half-life has been identified. These transport studies have been extended during the last year. Changes in transport in the auditory nerve of rapidly transported glycoproteins have been found after destruction of hair cells. Differences have been found and studied between rapidly transported optic nerve and auditory nerve proteins.

During the past year, structural and developmental studies have been carried out on the normal and neurologically mutant mouse cochlear nucleus and been submitted for publication. Cell birth dates in the mouse cochlear nucleus were determined using tritiated thymidine autoradiography. Nine cell types were identified in the Nissl stained cochlear nucleus of the normal mouse. Three major birth dates during the gestational period (days 10, 12 and 14) were found, corresponding to the birth of large cells, medium cells and small cells respectively. Cell types of the normal and neurologically lesioned mutant mouse, Reeler, were compared. The main effects of the lesion were found to be the disruption of the laminated organization of the dorsal cochlear nucleus and a 30% decrease of the total number of granule cells. The distribution of acetylcholinesterase-positive fibers in the cochlear nucleus of the normal and the Reeler mouse was compared. The major difference was found to be that although acetylcholinesterase-positive fibers do innervate the nucleus in the Reeler, they are not able to establish normal contact with the granule cells.

The studies concerning cochlear nucleus projections that were mentioned in last year's report have been published. The results suggest that there are symmetrical crossed connections between the ventral cochlear nuclei bilaterally that course through the dorsal and intermediate striae. Described in the publications are also important technical improvements of techniques using the horseradish peroxidase reaction product for visualization. An important improvement of the Golgi method has also been published this year from the Laboratory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02216 05 LNO															
PERIOD COVERED October 1, 1970 to September 30, 1980																	
TITLE OF PROJECT (80 characters or less) Inner Ear Neuronal Mechanisms: A Multidisciplinary Analysis																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																	
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: J. Fex</td> <td style="width: 33%;">Chief, LNO</td> <td style="width: 33%;">LNO NINCDS</td> </tr> <tr> <td>R. A. Altschuler</td> <td>Staff Fellow</td> <td>LNO NINCDS</td> </tr> <tr> <td>D. W. Hoffman</td> <td>Staff Fellow</td> <td>LNO NINCDS</td> </tr> <tr> <td>M. R. Martin</td> <td>Senior Staff Fellow</td> <td>LNO NINCDS</td> </tr> <tr> <td>R. J. Wenthold</td> <td>Senior Staff Fellow</td> <td>LNO NINCDS</td> </tr> </table>			PI: J. Fex	Chief, LNO	LNO NINCDS	R. A. Altschuler	Staff Fellow	LNO NINCDS	D. W. Hoffman	Staff Fellow	LNO NINCDS	M. R. Martin	Senior Staff Fellow	LNO NINCDS	R. J. Wenthold	Senior Staff Fellow	LNO NINCDS
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LAB/BRANCH Laboratory of Neuro-otolaryngology																	
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SUMMARY OF WORK (200 words or less - underline keywords)																	
<p>The long-range purpose of the project is to study the biochemistry, morphology, pharmacology and physiology of inner ear neurons and other cells and to describe the mechanisms of their interactions.</p> <p>1. <u>Two-dimensional electrofocusing/electrophoresis</u> has been applied to the analysis of <u>cochlear proteins</u> after <u>in vivo labeling</u> and may provide a <u>very sensitive approach</u> of the study of cochlear function. 2. <u>Enkephalin-like immunoreactivity</u>, visualized as <u>immunofluorescence</u>, has been demonstrated in the two <u>olivocochlear</u>, <u>efferent neuronal systems</u> of the <u>organ of Corti</u> in guinea pig and cat. <u>No such immunoreactivity</u> was found in <u>hair cells</u> of the organ of Corti, or in the <u>auditory nerve</u>. 3. <u>DL-α-amino adipate</u> in the <u>cochlea</u> at high concentrations does not change click evoked auditory nerve compound action potentials. This implies that the postsynaptic receptor of the <u>hair cell-auditory nerve synapse</u> is <u>not</u> of the N-methyl-D-aspartate, <u>aspartate-preferring type</u>.</p>																	

Project Description:

Objectives: To study the biochemistry, morphology, pharmacology and physiology of inner ear neurons and other cells and their interactions and to describe the mechanisms of these interactions.

The following subprojects are now serving these objectives:

I. A study of proteins of the cochlea of normal and genetically deaf animals.

II. An immunocytochemical study of the distribution of small peptides and proteins in the cochlea of mammals.

III. A study of the effects of excitatory amino acid antagonists on sound evoked activity in the auditory nerve.

Methods Employed:

I. As described in the previous report, cochlear proteins were labeled by exchanging the perilymph with a solution containing ^3H - or ^{35}S -labeled amino acid or fucose. The cochlea was then dissected under fluid and the proteins analyzed by one-dimensional gel electrophoresis or two-dimensional electrofocusing/electrophoresis. Labeled proteins were detected by fluorography. Endolymphatic surface proteins of the stria vascularis were labeled after dissecting, in buffer, strips of spiral ligament with the stria vascularis attached. The surface proteins were then labeled by lactoperoxidase-catalyzed iodination. The tissue was washed and the stria vascularis was dissected from the spiral ligament and then analyzed.

II. Antibodies to methionine enkephalin were generated in rabbits using methionine enkephalin coupled with glutaraldehyde to bovine thyroglobulin. Guinea pigs and cats were anesthetized and transcardially perfused with fixation fluid. The cochleae were removed and locally perfused with the same fixation fluid, rinsed and prepared for surface preparations of the organ of Corti or for cryostat sections of the cochlea. Modifications of the indirect fluorescence technique of Coons were used on both preparations. After incubation with the antiserum against methionine enkephalin, FITC-labeled swine anti-rabbit immunoglobulin antiserum was used for the second incubation. The final preparations were viewed with a Zeiss photomicroscope under epifluorescent illumination. Routine specificity studies were carried out.

III. The experiments were carried out on cats which were anaesthetized with pentobarbital sodium (40 mg/kg intraperitoneally with maintenance doses of 10-15 mg/kg). The cisterna magna was opened and the head of the animal tilted, with the round window of the cochlea facing upward to minimize the flow of cerebrospinal fluid through the cochlear aquaduct into the tympanic scala. The bulla was opened and most of the membrane of the round window of the cochlea was removed by cauterization to avoid bleeding. Cochlear

potentials were recorded from the rim of the round window with a platinum-iridium wire electrode, 0.1 mm in diameter. As a control that conditions were stable before DL- α -amino adipate (DLAA) was applied, a series of cochlear potentials were recorded between repeated substitutions of perilymph in the basal turn of the tympanic scala with artificial perilymph (140 mM NaCl, 12.5 mM NaHCO₃, 3.5 mM KCl, 1.3 mM CaCl₂, 1.14 mM MgCl₂, and 3.4 mM glucose: bubbled with 95% O₂ and 5% CO₂ at 38-40°C; transferred to ice and reheated to 38-40°C immediately before use). DLAA was added to the artificial perilymph to concentrations of 1.0 and 10.0 mM.

Major Findings:

I. It was shown that one and two dimensional electrophoretic analysis of cochlear proteins after in vivo labeling may provide a very sensitive approach to the study of cochlear function.

With the two dimensional analysis, more than 200 polypeptides were resolved. Seven polypeptides exposed on the endolymphatic surface of the stria vascularis were identified by the lactoperoxidase-catalyzed iodination. No consistent changes in the protein patterns of the stria vascularis from the waltzing guinea pig were detected.

II. In the cochlear osseous spiral lamina, immunofluorescence was localized to unmyelinated fibers of the intraganglionic spiral bundle. In the organ of Corti, immunofluorescence was localized to a small number of fibers at inner hair cells, the inner spiral bundle, tunnel spiral bundle, tunnel crossing fibers at the level of the tunnel floor, to an occasional spiral outer fiber and to the synaptic region of outer hair cells in the three rows of the basal turn of the cochlea. Less immunofluorescence was found in the outer hair cell region towards the apex, with none seen at the apex. At the most apical region the inner spiral bundle became patchy and the tunnel spiral bundle developed arcades. No immunofluorescence was found in spiral ganglion cells, in auditory nerve fibers or in hair cells of the organ of Corti. Generally, the findings were the same in cat as in guinea pig, the latter being studied more in detail. Thus, in these two mammals, enkephalin-like immunoreactivity was found in structures of the two efferent systems of the organ of Corti and in no afferent structures.

III. DL- α -amino adipate, locally applied to the cat cochlea at concentrations of 1 mM and 10 mM, has no effect on the amplitude, latency or shape of click evoked auditory nerve compound action potentials at 5, 10 and 40 dB above threshold.

Significance to Biomedical Research and the Program of the Institute:

This multidisciplinary study on inner ear structures and mechanisms, including the sensory cells and the neurons and their interactions, provides new knowledge on the poorly understood mechanisms of hearing. Such knowledge is of direct significance to biomedical research, will lead to better understanding of the causes of sensory deafness and nerve deafness and will most

likely lead to better management of hearing disorders.

In particular, referring to subprojects:

I. The present results showed that one and two dimensional electrophoretic analysis of cochlear proteins after in vivo labeling may provide a very sensitive approach to the study of cochlear function. This approach may be especially useful in elucidating the molecular mechanisms underlying such disorders as ototoxicity and genetic hearing abnormalities. Although in the present study about 200 spots were detected with two dimensional electrophoretic analysis, additional minor polypeptides can be detected by expanding the electrofocusing gradient and developing the fluorographs longer.

II. The results lead to the conclusion that olivocochlear efferent nerve fibers and terminals in the organ of Corti contain enkephalin-like immunoreactivity. It has previously been shown that olivocochlear efferents are likely to be cholinergic. Thus, it is also likely that such efferents in the organ of Corti contain and release both acetylcholine and enkephalin when activated.

III. It could be concluded that the postsynaptic receptor of the hair cell-auditory nerve synapse is not of the N-methyl-D-aspartate, aspartate-preferring type.

Proposed Course:

Preliminary studies show that two dimensional electrofocusing/electrophoresis can also be applied to the study of proteins of the organ of Corti. Whether such studies will be carried out during the next fiscal year is as yet not settled.

The findings of enkephalin-like immunoreactivity in the organ of Corti will be followed up. We are planning to search for opiate receptors in the organ of Corti. If present, the distribution of such receptors will be mapped. If the type of receptor can be determined, this will lead to pharmacological experiments. We are planning to use immunocytochemical techniques for showing the distribution of function-related proteins in the organ of Corti. We believe that this Laboratory is the first to have successfully applied immunocytochemistry at light microscopy level for studies of the organ of Corti. This approach seems very promising and exciting.

Publications:

Fex, J. and Martin, M. R.: Lack of effect of DL- α -amino adipate, an excitatory amino acid antagonist, on cat auditory nerve responses to sound. Neuropharmacology. In press.

Gulley, R. L., Fex, J. and Wenthold, R. J.: Uptake of putative neurotransmitters in the organ of Corti. Acta Otolaryngol. 88: 177-182, 1979.

Lee, K. S. and Drescher, D. G.: Derivatization of cysteine and cystine for fluorescence amino-acid analysis with the o-phthalaldehyde/2-mercaptoethanol reagent. J. Biol. Chem. 254: 6248-6251, 1979.

Wenthold, R. J. and McGarvey, M. L.: Analysis of proteins in the stria vascularis of the normal and the waltzing guinea pig. Acta Otolaryngol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02217 05 LNO
PERIOD COVERED October 1, 1970 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Synaptic Transmission and Neuronal Connections of the Mammalian Cochlear Nucleus		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
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<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of the project is to study the biochemistry, morphology, pharmacology and physiology of synaptic transmission and neuronal connections of nerve cells of the mammalian cochlear nucleus. New results from the following studies give increasing support for a <u>neurotransmitter role for glutamate and aspartate in the cochlear nucleus</u> : 1. <u>Corresponding to terminals of the auditory nerve, rings of aspartate aminotransferase-like immunoreactivity have been found around cells in the ventral cochlear nuclei.</u> 2. <u>The effects of iontophoretically-applied antagonists on auditory nerve and amino acid evoked excitation of anteroventral cochlear nucleus neurons.</u> 3. <u>Glutaminase and aspartate aminotransferase decrease in the cochlear nucleus after lesion of the auditory nerve.</u> Furthermore, 4. <u>the first characterization of proteins in the auditory nerve through a study of axonal transport has been published; the study is being continued.</u> 5. <u>Structural and developmental studies of cochlear nucleus of normal mouse</u> has been carried out. 6. <u>The cochlear nucleus of mutant Reeler mouse</u> has been studied. 7. <u>Studies on connections of the cochlear nucleus, superior olivary region and inferior colliculus have been published.</u>		

Project Description:

Objectives: To study the biochemistry, morphology, pharmacology and physiology of synaptic transmission and neuronal connections of nerve cells in the mammalian cochlear nucleus.

The following subprojects are now serving these objectives:

I. Synaptic transmission. Morphology.

a. Immunocytochemical localization of aspartate aminotransferase-like immunoreactivity in the guinea pig cochlear nucleus at the light microscopy and electron microscopy level.

b. Immunocytochemical localization of enkephalin-like immunoreactivity in the guinea pig cochlear nucleus.

II. Synaptic transmission. Physiology/pharmacology.

a. Effects of excitatory amino acids and their antagonists on neurons and auditory nerve transmission in the cochlear nucleus.

b. Effects of inhibitory amino acids and their antagonists on neurons and auditory nerve transmission in the cochlear nucleus.

III. Synaptic transmission. Biochemistry.

Immunocytochemical localization of aspartate aminotransferase in the cochlear nucleus.

IV. Axonal transport in the auditory nerve.

V. Structural and developmental studies.

a. Morphology of the cochlear nucleus of the normal and reeler mutant mouse.

b. Histogenesis of the cochlear nucleus of the mouse.

c. Acetylcholinesterase positivity in the cochlear nucleus of normal and reeler mutant mouse.

VI. Connections of the cochlear nucleus.

a. Cochlear nucleus projections.

b. Cells and connections of the superior olivary region.

c. Inferior colliculus projections.

d. Technical advances in histochemistry related to cell morphology.

Methods Employed:

I. a. Antibodies were generated against aspartate aminotransferase (AAT) and the antisera characterized. These were then used in immunocytochemical studies on the guinea pig cochlear nucleus. Indirect immunofluorescence techniques were used on cryostat sections for light microscopic visualization. Unilateral eighth nerve lesions were carried out and the cochlear nucleus examined 2, 3 and 7 days post lesion. Immunoelectron-microscopic techniques were developed for ultrastructural localization of AAT-like immunoreactivity in the rostral AVCN. The peroxidase-anti-peroxidase (PAP) technique was used on vibratome sections of aldehyde fixed brains.

b. The study of met-enkephalin-like immunoreactivity in the rat dorsal cochlear nucleus, described in last year's Annual Report, was expanded to the cochlear nucleus of the guinea pig. The same indirect immunofluorescence techniques were utilized. Colchicine pre-treatment was similarly necessary in order to concentrate the neuropeptide in cell bodies to allow its visualization. This was done by applying 10 µg of colchicine in 10 µl of PBS directly over the dorsal cochlear nucleus, exposed by opening the cranium and removing a portion of the cerebellum.

II. a. and b. The methods employed were described in last years Annual Report and were shortly as follows. Experiments were performed in a sound chamber on anesthetized cats. Action potentials of single units were recorded extracellularly from the anterior and posterior divisions of the anteroventral cochlear nucleus using the 4 M NaCl-filled center barrel of seven barrel microelectrodes. Five of the six outer barrels contained the compounds to be ejected electrophoretically using standard procedures as described by Curtis, 1964. In the brainstem auditory system, those cells that receive large calyceal endings show a peculiar waveform associated with unit action potentials. Preceding each action potential by approximately 0.5 msec is a "prepotential" that has been shown to be presynaptic. The presence or absence of prepotentials were used for categorizing unit types. The rate of unit discharge was computed and displayed on a potentiometric chart recorder for on-line assessment of drug action. Averages and histograms were made on-line with a PDP-11 computer, records were kept on magnetic analog tape.

III. Antibody to AAT was made using the commercially available cytoplasmic form of the enzyme obtained from pig heart. This preparation was purified once on a Sephadex G 150 column. Fractions containing AAT activity were pooled and used for the production of antibodies in rabbits. Antibodies obtained following this method reacted with both pig heart AAT and guinea pig brain AAT. To insure that antibodies were specific for AAT, our efforts were directed at obtaining a homogeneous preparation of AAT. The only satisfactory method was found to be extraction of AAT from SDS polyacrylamide gels. This extracted preparation cross-reacts with our antibody. This purified preparation was used for the preparation of antibodies in rabbits, subproject Ia.

IV. Methods for axonal transport studies in the auditory nerve were given in last year's report. Three hours after injection of labeled amino acid in the cochlea has generally been used for analyzing rapidly transported proteins in the cochlear nucleus. Optic nerve proteins were labeled by injecting 500 μ Ci 35 S-methionine or 500 μ Ci 3 H-fucose in 5 μ l PBS into the vitreous body of one eye of an anesthetized guinea pig. The superior colliculus was dissected (usually 6 h after injection) and analyzed.

The interaction between rapidly transported proteins and immobilized lectins was studied using 35 S-methionine labeled proteins. Three labeled superior colliculi or 4 labeled cochlear nuclei were solubilized with SDS and applied to columns of immobilized concanavalin A or wheat germ agglutinin. Bound glycoproteins were eluted with α -methyl-d-mannoside or N-acetylglucosamine. Samples were dialyzed and concentrated for analysis on gels.

Subfractionation of superior colliculus and cochlear nucleus synaptic plasma membranes was done after 35 S-methionine injection. Synaptic plasma membranes were obtained using the method of Jones and Matus (BBA 356, 276) or Gurd et al. (J. Neurochem. 22, 281). Synaptosomes were obtained using the method of Gurd et al. Externally oriented proteins or synaptosomes were labeled using lactoperoxidase-catalyzed iodination.

Hair cells were destroyed by injecting 250-300 guinea pigs with neomycin sulfate (250 mg/kg/day) for 8 days.

Samples were analyzed with one and two-dimensional electrophoresis. One dimensional electrophoresis was done on gradient gels (5-17% or 7-17%) and two dimensional gels were done on 10% acrylamide. Gels were analyzed by fluorography.

V. a. Cell types in the cochlear nucleus of the mouse were differentiated using a cresyl violet/luxol fast blue stain on 10 μ m thick paraffin embedded sections which had been fixed in Bodian's fixative. Cell types were characterized by shape and size of the cell body, distribution and appearance of the Nissl substance, the size, shape and location of the nuclei, and the location within the nucleus.

Material from Reeler mutant mice, and their normal littermate, as controls, were prepared and cell types differentiated using the procedures and criteria described above. The distribution and number of granule cells in the cochlear nucleus of normal and Reeler mutant mice were estimated using a computer based system interfaced with a microscope stage. The program stored data concerning the total number of granule cells within any 130 μ m² area of a section through the cochlear nucleus. This program gives information about differences in estimated total number of granule cells, their density and distribution. The program is adaptable to any cell type in the cochlear nucleus that one might choose to quantify in these terms.

b. Cell birth dates were determined using tritiated thymidine autoradiography. Animals were injected with the labeled compound, 5 μ Ci/g body weight intraperitoneally, at half day intervals between gestation days 5 and 19.5 and on postnatal days 2, 4, 6, 8, 10, 12 and 14. Fetuses of gestation day 8.5, 9.5 and 10.5 were injected using a laparotomy procedure. Animals were killed at 21 days postnatal and fixed in iced Carnoy's solution, paraffin embedded and cut as 6 μ m thick sections. Mounted sections were dipped in K₂ emulsion (3 μ m thick) and developed after 6 weeks in D19B. Sections were then stained with Erlich's hematoxylin.

c. Cholinesterase activity in the cochlear nucleus of the mouse was demonstrated using the direct coloring method of Karnovsky and Roots. Animals were fixed in 20% formalin in isotonic sodium sulphate at 37°C. Brains were stored in 10% formalin in isotonic sodium sulfate at 4°C for 6-18 hours before being transferred to a 20% alcohol solution, also at 4°C. The tissue was stored for an additional 6-24 hours and then frozen sections were cut at 40 μ m. Sections were incubated in the Karnovsky and Roots solution for 90 minutes at 37°C. The sections were then mounted and sometimes counter-stained with cresyl violet. In control experiments BW 284C 51 was used in the incubate to inhibit true acetylcholinesterase and ethopropazine HCl to inhibit pseudo-cholinesterases.

VI. The methods employed for the different aspects of this subproject have been described in Annual Reports of previous years, some have been published previously and, of the remaining, almost all have been described in publications appearing this year (see Publications). The cytological study of cells and connections of the superior olivary region has included Golgi, Nissl and Protargol material and the previously described use of a computer-controlled microscope stage.

Major Findings:

I. a. Immunofluorescence techniques showed rings of AAT-like immunoreactivity around cells in the ventral cochlear nuclei. These correspond to auditory nerve terminals as previously demonstrated in this Laboratory by anterograde transport autoradiography. After eighth nerve lesions such rings were no longer seen in the ipsilateral cochlear nucleus. Instead only a few small patches of immunofluorescence could be seen around the cells. At the ultrastructural level in the rostral AVCN we were able to demonstrate AAT-like immunoreactivity on bushy cells in terminals containing large round vesicles and exhibiting other morphological characteristics of auditory nerve terminals.

b. The pattern of met-enkephalin-like immunoreactivity in the guinea pig is similar to that previously described in the rat (last year's Annual Report), with immunoreactivity localized in small cells in the deep layer of the dorsal cochlear nucleus. Immunoreactive fibers and terminals were seen in the postero- and anteroventral cochlear nucleus as well as in the dorsal cochlear nucleus. Fibers were also seen in the dorsal and intermediate striae.

II. a. The manner in which anteroventral cochlear nucleus units respond to iontophoretically applied compounds is not dependent on their characteristic frequency or response pattern. Single units can be separated according to their waveform as prepotential and non-prepotential. Prepotential units normally either do not respond or show depolarization block to excitants. DL- α -aminoadipate, D- α -aminoadipate, DL- α -aminosuberate, HA966 and magnesium ions are all effective in blocking the auditory nerve evoked activity of these units. Glutamate diethyl ester however rarely affects this response. Non-prepotential units readily respond to glutamate, aspartate, kainate and N-methyl-D-aspartate, and occasionally to acetylcholine. DL- α -aminoadipate blocks N-methyl-D-aspartate responses, reduce the glutamate and aspartate responses, and does not affect kainate or acetylcholine responses. DL- α -aminoadipate rarely affects low level tone burst evoked responses but does consistently reduce responses when tone bursts of 20 dB above threshold are used. D- α -aminoadipate, DL- α -aminosuberate, HA966 and magnesium also reduce this response which again is rarely affected by glutamate diethyl ester. On the same units antagonists which block synaptic transmission block N-methyl-D-aspartate responses and preferentially reduce aspartate over glutamate responses. Glutamate diethyl ester does not affect N-methyl-D-aspartate and preferentially reduces glutamate over aspartate responses. The data offers evidence that aspartate is more likely to be the transmitter of the auditory nerve than glutamate.

b. Studies on inhibitory amino acids and their antagonists are in a preliminary stage. Receptors for both glycine and GABA are present on neurons in the anteroventral cochlear nucleus. However, the antagonists strychnine and bicuculline hydrochloride do not appear to have a major effect on the form of the response of these neurons to tone bursts at their characteristic frequency.

III. Results from biochemical steps taken to produce an antibody to aspartate aminotransferase are described under I. a.

IV. Two-dimensional analysis of ^{35}S -methionine labeled rapidly transported proteins show at least 6 polypeptides are present in the superior colliculus (SC) that are not present in the CN and 2 in the CN not present in the SC. After ^3H -fucose injection at least 8 polypeptides are present in SC and not found in CN and at least 6 in CN not found in SC. The differences after ^{35}S -methionine injection involve polypeptides that are glycoproteins as determined with fucose labeling and separation on immobilized lectins. Five polypeptides with isoelectric points 4.8-5.4 and molecular weights 90K-140K are labeled in both CN and SC but in greatly different amounts. In the CN a 140K polypeptide is predominant and is one of the major rapidly transported proteins in the auditory nerve. The 140K polypeptide is minor in the SC while 110K and 130K polypeptides are more heavily labeled. These polypeptides turn over rapidly in both SC and CN; the 140K polypeptide has a half-life of less than 3 hours in CN. All are glycoproteins based on fucose incorporation and lectin binding and all subfractionate with the synaptic

plasma membrane. These 5 polypeptides appear to be the most rapidly turning over rapidly-transported proteins in both SC and CN. It is clear from these studies that proteins of the SPM do not turn over uniformly, but rather exhibit a range of half-lives.

To further characterize rapidly transported proteins, synaptosomes were prepared from SC and externally oriented proteins were labeled with lactoperoxidase-catalyzed iodination. One of the major iodinated polypeptides co-migrates with a rapidly transported glycoprotein found in SC but not CN. If the iodinated proteins are separated on Con A columns, this protein is bound, further suggesting it is identical to the rapidly transported glycoprotein. None of the five rapidly-turning-over glycoproteins, described earlier, is labeled.

Two or 4 weeks after neomycin treatment or in waltzing guinea pigs (10-90 days old) two rapidly transported polypeptides are present in amounts (based on incorporation of ^{35}S -methionine) greater than those in normal auditory nerves. These polypeptides with molecular weights of 30K and 40K are both glycoproteins based on fucose incorporation. The changes in the 40K polypeptide were quantitated by cutting the spot out of the dried gel. Two weeks after neomycin treatment, the amount of this protein had increased 400%. Similar changes were also seen in the waltzing guinea pig.

V. a. Nine cell types were identified in the Nissl stained cochlear nucleus of the normal mouse. In all but three respects the cell types correspond to those seen in the cat. The large spherical cells of the anteroventral cochlear nucleus of the cat, which are associated with low-frequency tone burst response, are absent in the mouse. This is compatible with the stress placed on processing high frequency sound information in the cochlea of the mouse. Unlike the ghost-like appearance of octopus cells in the cat, the cells in the caudal posteroventral nucleus stain very heavily for Nissl substance. In the pyramidal cell layer of the dorsal cochlear nucleus there are two large cell types in the mouse compared to one in the cat. The first is the typically large bipolar pyramidal cell and the second is a somewhat smaller and more ovoid cell, referred to as the horizontal, with the primary dendrites oriented parallel to the surface of the nucleus. The significance of this difference is not yet apparent.

There are no gross abnormalities apparent in the cells of the ventral cochlear nucleus in the Reeler mutant. The main effect of the lesion is a disruption of the laminated organization of the dorsal cochlear nucleus and a 30% decrease in the total number of granule cells. The loss is not uniform. The granule cells on the lateral wall of the ventral nucleus are particularly sparse. The extension of this lesion to the cochlear nucleus is not surprising since this nucleus and the cerebellum which is severally affected in the same way in this mutant develop from the same embryonic region. Reelers are not deaf and the finding of this lesion raises questions about the development and processing of auditory information by the cochlear nucleus.

b. There are three major birth dates during the gestational period; days 10, 12 and 14. These correspond to the birth of large cells, medium cells and small cells respectively. Pyramidal cells, giant cells and dark-staining cells are born on day 10, the horizontal cell of the pyramidal layer, globular cells, multipolar cells and spherical cells are born on day 12 and the small cells and granule cells are born on day 14. It is worth noting that the two cell types (other than granule cells) found in the pyramidal layer of the dorsal cochlear nucleus are born on separate days, 10 and 12. A second wave of cell growth occurs postnatally with a peak at day 2 and continuing through day 14 corresponding to the birth of glial cells, particularly oligodendrocytes.

c. In the normal mouse acetylcholinesterase-positive fibers enter the cochlear nucleus from its caudomedial surface. There is a prominent projection along the granule cell bridge between the dorsal and ventral nuclei to the lateral surface. A few fibers from this bundle enter the deep dorsal cochlear nucleus and eventually reach the pyramidal cell layer. These fibers do not extend to the granule layer along the lateral surface of the ventral nucleus. Acetylcholinesterase positive fibers reach these cells by passing through the posteroventral nucleus where they give off numerous collaterals. The interstitial region and the most postero-medial part of anteroventral nucleus also receive numerous fibers.

In the Reeler mutant the bundle passing along the granule cell bridge between the dorsal and ventral nuclei is present in a reduced state and does not extend to the lateral edge. No fibers enter the dorsal cochlear nucleus. Granule cells on the lateral wall of the ventral nucleus do not receive acetylcholinesterase positive fibers. However, the innervation of the remainder of the ventral nucleus appears to be similar to that in the normal animal. Thus, although acetylcholinesterase-positive fibers do innervate the nucleus in this mutant they are not able to establish normal contacts with the granule cells.

VI. In the past year, the studies within this subproject, all interconnected, have moved forward. Last year's Annual Report described the study, then recently published from the Laboratory, on the identification of different cell classes that send projections to the inferior colliculus, including preolivary cells. Previously, preolivary cells were not known to have ascending projections. To enable a full description of the connections of the superior olivary region with the cochlear nucleus and the cochlea on one hand and with higher centers on the other hand the cytological atlas of the superior olivary region has been prepared. The physical production of the atlas has been carried out and it is now being prepared for publication. At least two publications will result from the effort: a cytological study and the atlas.

Experimental evidence has been provided that axons of some preolivary cells project to both sides, perhaps to both cochleae and both cochlear nuclei.

Significance to Biomedical Research and the Program of the Institute:

Although glutamate and aspartate for many years have been suggested to be neurotransmitters, this has not been proven. One major drawback has been the lack of a specific method for identification of neurons using these substances as neurotransmitters. One of the goals of our work on the auditory nerve has been to fully characterize a possible glutamergic/aspartergic neuron and apply these findings to identify and characterize other neurons using glutamate and aspartate as neurotransmitters. The immunocytochemical studies make two major points: 1. They provide a direct visualization of AAT in terminals of the auditory nerve. Although the biochemical evidence strongly suggested this enzyme and glutamate and aspartate are concentrated in terminals and fibers of the auditory nerve, biochemical studies cannot be used to precisely locate these substances. 2. These studies raise the interesting possibility that AAT may serve as a marker for neurons using glutamate or aspartate as a neurotransmitter. Preliminary electron microscopic studies show that very likely the only terminals in the anteroventral cochlear nucleus of the guinea pig with AAT immunoreactivity are the terminals of the auditory nerve.

These new results and the findings under II. a., together with the previous data from this Laboratory, make the auditory nerve synapse the best characterized putative glutamate aspartate synapse in the mammal. Our identification of this system in which glutamate or aspartate may function as neurotransmitters represents a major advance in biomedical research.

The brain's endogenous opiates have been in the focus of intensive studies in laboratories all over the world during the last few years. Our results that the auditory system at the level of the cochlear nucleus (and the cochlea) receives neurons with enkephalin-like immunoreactivity is of great interest. However, the significance of and function of opiates in neurons is not yet understood.

Our findings that changes in rapidly transported proteins occur in the auditory nerve after hair cell loss show that protein synthesis in a neuron can be influenced by synaptic inputs. Before we can determine why these two proteins change, it will be necessary to determine their normal roles. For example, their increase may be a very early step in the degeneration of these neurons.

The study of axonal transport shows major differences exist in rapidly transported proteins in the auditory and the optic nerves. It is unclear yet what the role of these proteins, present in one neuron and not in others, may be. All these proteins appear to be associated with the synaptic plasma membrane. Some proteins may be associated with the neurotransmitter and be dependent on the substance used for the neurotransmitter. Another possibility is that some of these proteins are instrumental in synaptic development and maintenance. Many of the proteins are glycoproteins and some can be demonstrated to be on the external surface of the membrane.

The Laboratory has continued to provide new data on the organization and development of the cochlear nucleus that have to be considered for the understanding of the function of the auditory nerve in health and in disease.

Our computer model for assembling, evaluating and displaying a vast number of interrelated morphological data points is of importance for studies of complicated neuronal systems. Our model has been applied to the superior olivary region, describing its nuclei and their connections and projections. The model is applicable to any similar system.

Proposed Course:

The study through immunohistochemical methods of the localization at the cellular and subcellular level of enzymes related to the metabolism of glutamate and aspartate will be continued. The electrophoretic pharmacological/physiological studies of cells receiving auditory nerve synapses in the cochlear nucleus will be continued. An attempt to define inhibitory input to such cells will be made.

The study of proteins transported in the axons of the auditory nerve will be continued. Attempts will be made towards clarifying which proteins may be directly associated with neurotransmission and which proteins may be instrumental in synaptic development and maintenance.

A major effort in the studies of axonal transport will be directed at characterizing some of the specific rapidly transported proteins. We will investigate whether a synaptic plasma membrane preparation is a sufficient starting material for purification of these proteins. If these proteins can be purified to homogeneity, antibodies to them can be produced and used to localize these proteins throughout the nervous system. This will also lead to a study of the biosynthesis of these substances.

The study of connections of the cochlear nucleus will be continued including attempts to show whether auditory activity in the one cochlear nucleus may suppress auditory activity in the contralateral cochlea nucleus.

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

National Institute of Infectious Diseases Branch
Neurological and Communicative Diseases and Stroke

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ANNUAL REPORT

October 1, 1979 through September 30, 1980
Infectious Diseases Branch, IRP
National Institute of Neurological and
Communicative Disorders and Stroke

John Louis Sever, M.D., Ph.D., Chief

I. RESPONSIBILITY OF THE BRANCH

The responsibility of the Infectious Diseases Branch is to carry out planned, coordinated research programs concerned with infections which damage the human nervous system. The Branch is divided into four sections: 1) Immunochemistry and Clinical Investigations; 2) Experimental Pathology; 3) Neurovirology; and 4) Electron Microscopy. These sections utilize the techniques of immunology, clinical investigations including human volunteers and clinical trials, experimental pathology with nonhuman primates, virology, bacteriology, mycoplasmaology, neurovirology, human tissue culture and electron microscopy.

II. PROGRAM SEGMENTS

The program segments are: a) perinatal; b) acute; and c) chronic. In each segment we are concerned with: 1) etiology and diagnosis; 2) treatment; and 3) prevention.

The research areas in the program segments include:

A. Perinatal

Develop and utilize large scale methods to study the relation between viral, bacterial, mycoplasma and protozoa infections in the perinatal period and birth defects, related abnormalities and pediatric neurological diseases. Investigate approaches to early diagnosis, treatment and prevention using combined laboratory and clinical studies.

B. Acute

Investigate agents which may be responsible for acute neurological diseases such as meningitis, encephalitis, Reye's syndrome, Bell's Palsy, and tic douloureux as well as possible methods for rapid diagnosis, treatment and prevention.

C. Chronic

Study chronic neurological diseases such as multiple sclerosis, amyotrophic lateral sclerosis, progressive multifocal leukoencephalopathy, Parkinson's disease, subacute sclerosing panencephalitis, Alzheimer's and Pick's disease and epilepsy using combined tissue culture, immunological, serological, genetic, electron microscopic and clinical approaches for possible infectious etiologies. Whenever possible, explore methods for early diagnosis, treatment and prevention.

III. SECTION ACTIVITIES

A. Section on Immunochemistry and Clinical Investigations (ICI)

1. Perinatal

The Section is responsible for the research and the analysis of Collaborative Perinatal Project sera and data for infection in 60,000 pregnancies. The approaches being used include: 1) clinical infections - correlation with pregnancy outcomes; 2) serological investigation of 8,000 abnormal and 8,000 controls; and 3) high IgM among 30,000 children as a method to identify infected children. Highly sensitive ELISA tests are being applied to these studies.

Additional studies include high risk children and infections in relation to neonatal deaths and herpesvirus infections in pregnancy. A study is being conducted to determine the rate of herpes infections in middle class pregnant women.

2. Acute

The ELISA tests are being used in studies of CSF and serum patients with a number of different neurological diseases. Group B streptococcal meningitis infections are being studied in patients at George Washington University Hospital and in experimental monkeys in our laboratories. Treatment protocols with new antiviral agents are being investigated.

3. Chronic

Oligoclonal IgG have been found in the CSF of patients with several different types of neurological diseases including MS and myasthenia gravis. Specific tests for antibody are in progress using the new micro oligoclonal method. Special serological investigations of MS and ALS patients are in progress.

Fundamental studies of virus induced demyelination with mouse hepatitis virus are being conducted.

Using a new Flow Cytofluorograph technique studies are underway to define the immune responses in MS and other neurological diseases.

B. Section on Experimental Pathology (EP)

1. Perinatal

This Section is conducting studies of experimental monkeys which develop CNS and other damage when infected in utero or in the newborn period. Current agents include CMV and VEE viruses. The value of new chemotherapeutic materials is under investigation. Experimental treatments for congenital toxoplasmosis and herpesvirus infections are being studied in monkeys.

2. Acute

Group B streptococcal meningitis is being studied in monkeys and new methods for treatment and prevention are being tested. Acute encephalitis with a varicella like virus (The Delta Agent) and herpes encephalitis is being studied.

3. Chronic

Studies of progressive multifocal leukoencephalopathy in monkeys are in progress. Inoculation by various routes and with immunosuppression is being studied.

Herpes zoster (shingles) is under investigation in experimental animals. Super-infection with a second virus and reactivation of latent infection is being tested.

C. Section on Neurovirology (NV)

1. Perinatal

This Section is studying new strains of CMV and mechanisms of genetic control. Cellular and humoral responses are being investigated in monkeys for both CMV and herpes.

2. Acute

Cellular immune studies are being conducted for patients with acute herpesvirus, CMV and EBV infections.

3. Chronic

Mechanisms of cellular immunity to various viruses are being tested using patient material from individuals with MS, SSPE, EBV and other neurological diseases.

The pathogenesis of PML (JC virus) are under study with the Section on Experimental Pathology. Also, possible virus etiologies of MS are under investigation using a variety of cellular immune techniques.

The immune response of monkeys infected with CMV is being studied. New antiviral drugs are being tested in vitro and in experimental animals with chronic infection.

Mechanisms responsible for acute vs persistent infection are being analyzed using the simian hemorrhagic fever model. Approaches to clearing persistent infection are being studied.

D. Section on Electron Microscopy (EM)

This Section is using immunoelectron microscopy in studies of virus induced demyelination. Video intensification light microscopy along with freeze-fracture, scanning and transmission EM methods are being used with disassociated neuron cultures and Schwann cell cultures.

The mechanism of chronic infection in CNS tissue is being studied and the interaction between viruses and lymphocytes is being investigated. Membrane changes are being studied with measles, VSV mouse hepatitis and herpesviruses. The interaction between antibody and viruses is being tested for measles, and VSV viruses.

The motility and myelinating properties of cultured Schwann cells are being studied.

IV. FINDINGS

A. Perinatal

1. Detection of Low Levels of Rubella Antibody (ICI)

Our studies have shown that current methods for detecting low levels of rubella antibody are often inaccurate. The use of ELISA methods does not always clarify these problems and reference standards are needed to help diagnostic laboratories.

2. Commercial Kits for Rubella Antibody Often Vary in Sensitivity (ICI)

A study of 11 new kits marketed for rubella serology showed considerable variation in the ability to detect antibody to rubella. Some may give false negatives, others give false positives. The kits must be monitored carefully by the users.

3. Cytomegalovirus Antibody Does Not Protect Fetuses In Monkey Model (EP)

Rhesus monkey fetuses were susceptible to Rhesus CMV even though their mothers had antibody to CMV. This may be important in studies of vaccines for human CMV.

4. Venezuelan Equine Encephalomyelitis Virus causes Neurological Damage Within 10 Days After Monkey Fetuses Are Exposed (EP)

VEE candidate vaccine virus produced neurological damage which began 10 days after fetal monkeys were exposed in utero. The animals went on to develop cataracts, and hydrocephalus and other neurological damage.

B. Acute

1. Fetal Monkey Infection With Rhesus CMV (NV+EP)

Infection was induced in monkeys fetuses using Rhesus CMV in sero positive mothers. Neurological damage was evident. CNS antibody was produced.

2. Infection of Rat Schwann Cells With HSV-1 and VSV (EM)

Both HSV-1 and VSV were found to grow in rat Schwann cells. In the case of VSV viral protein synthesis abruptly stopped at 4 hours.

3. Chimps Harbor CMV Virus (EP)

A total of 14% of chimpanzees were found to harbor CMV virus in the urine and/or nasopharynx. This may interfere with studies of MS and other neurological diseases in these animals.

4. Experimental Herpes Encephalitis Produced (EP)

Experimental herpes encephalitis was produced in rhesus and cynomolgus monkeys. This provides models for the study of this disease.

5. Delta Agent (Varicella) Pneumonia and Encephalitis (NV)

The varicella model-Delta Agent in the Patas monkey was shown to produce pneumonia and encephalitis. This expands the utility of this model system.

6. Group B Strep In Newborn Protected by Penicillin to Mother or Child But Vaccine Not Effective (EP)

Studies of the group B Strep model in newborn monkeys showed the animals were partially protected by penicillin to the mother or child. This approach may be of value in human disease. A new vaccine developed for human use did not result in antibodies to this organism.

C. Chronic

1. Elimination of Persistent Infection by Superinfection (NV)

Persistent simian hemorrhagic fever (SHF) infection in patas monkeys was eliminated by superinfection with a related strain of SHF. This implies that immunoregulatory controls are extended to maintain persistent SHF virus infection in the patas and these controls can be over-ridden by superinfection with acute strains.

2. Acute and Chronic Strains of Simian Hemorrhagic Fever (SHF) Virus Found To Be Closely Related (NV)

Two strains of SHF virus which produce acute infection in the patas monkey and one strain which results in chronic infection were studied by polyacrylamide gel electrophoresis. Polypeptide composition of all three strains was similar and showed them to be closely related.

3. New Micromethod for Oligoclonal IgG (ICI)

A new technique which uses only 50 lambda of CSF was developed. This permits widespread use of this method for assisting in the diagnosis of MS and other diseases.

4. New Method for Study of Cellular Immune Reactions Using Small Numbers Of Cells (ICI)

A new Flow Cytofluorograph has been developed to identify immune responses in as little as 10^3 lymphocytes. This is being applied to studies of CSF lymphocytes.

5. Oligoclonal IgG in CSF of Patients with Progressive Myoclonus Epilepsy (ICI)

A study of CSF from Finnish Patients with Progressive Myoclonus Epilepsy showed that more than half had oligoclonal IgG. This indicates that IgG is being stimulated in the CSF.

7. MS Patients Have Immune Complexes and Associated Increased Viral Antibodies In The CSF (ICI)

Immune complexes were found in the CSF of MS patients and the levels correlated with the presence of viral antibodies in the CSF.

8. MS Bone Marrow Does Not Harbor Transmissible Agent (NV)

A report from England on a virus in the bone marrow of MS patients was not confirmed by our studies.

9. Abnormal Immunoregulatory Responses Found In MS Patients (NV)

MS patients had elevated suppressor T cells to measles virus in 30% of stable patients. Some patients failed to maintain normal suppressor activity to brain antigens.

10. Stable MS Patients Have Normal Immune Competence (NV)

Mitogenic and recall activity was found to be normal in stable MS patients. Patients who fail to have suppressor T cells to brain antigens develop cellular immunity to these antigens.

11. T Gamma Lymphocytes Participate In Antibody Directed Cytotoxicity (NV)

The T gamma lymphocyte subpopulation was shown to interact with IgG antibody to measles and directed killing of measles infected target cells.

12. Persistent Measles Infection In Mouse Neuron Cultures (EM)

Persistent measles infection was restricted to neurons in mouse spinal cord cultures. Nucleocapsids were formed but not incorporated into the virion although all viral proteins were expressed.

13. Comparison Between A Demyelinating (TS Mutant) and Wild Type Mouse Hepatitis Virus In The Nervous System (EM)

The TS mutant was expressed mainly in oligodendrocytes while the wild virus infected both neurons and glial cells. Both viruses produced intense fusion in vitro but there was more intense accumulation of intracytoplasmic inclusions in the case of the mutant.

This study provided an improved method for combining light microscopy and EM immunolabeling studies on unimbedded sections of fixed nervous tissue.

14. Schwann Cells Shown To Migrate and Pulsate Even After Intense Mitotic Stimulation In Vitro (EM)

Schwann cells were isolated, purified and stimulated to grow with cholera toxin and a pituitary factor. Video amplification microscopy showed that secondary cells had intense migratory and rhythmic pulsatory activities which might be related to their myelinating function.

15. Additional Monkeys Develop Brain Tumors Due to JC Virus (EP)

Three additional owl monkeys and one squirrel monkey developed brain tumors after inoculation with human JC virus.

16. Latent Varicella Like Delta Agent Activated In Patas Monkey (EP)

The latent varicella like Delta Agent virus was activated in Patas monkeys by superinfection with another virus. This provides a model for study of reactivation of this virus.

17. Myasthenia Gravis Patients have Oligoclonal Bands

Oligoclonal Banding of IgG in CSF of patients with myasthenia gravis suggest more extensive neurological involvement than was previously recognized.

18. Neurological Complications of Epstein Barr Virus Infections

Patients with EBV related neurological complications have been shown to have viral antibodies and oligoclonal IgG bands in CSF suggesting that viral antigens are expressed within the CNS.

19. Rabies Virus Transmitted by Corneal Transplantation: Second report.

Rabies virus can be transmitted from human to human by corneal transplantation. A second corneal transplant case of rabies encephalitis has occurred in Paris, France, suggesting the problem may be more prevalent than previously suspected.

20. Rubella Panencephalitis Following Required Rubella Infection

A second case of rubella panencephalitis occurred in a 14 year old male 12 years following acquired rubella infection. The incubation period for both acquired and congenital infections appears to be 12 years.

CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal year 1980

Bio Tech Research Laboratories Inc. (NO1-NS-8-2388)

TITLE: Provide Special Tissue Culture Cells and Reagents to NINCDS

Contractor's Project Director: Dr. Anton F. Stewen

Current Annual Level: \$47,500.00

Objective: This is a service contract to produce a variety of cells and reagents not available under other mechanisms for use in the research programs of the Branch.

Major Findings: A number of satisfactory lots of special tissue culture cells have been submitted to the branch for use in our studies of the JC virus in owl monkeys and the study of herpes, CMV and rubella virus in neurological disease.

Significance to the NINCDS Program and Biomedical Research: The cells and viruses produced by this Contract have been utilized in the research programs of the Branch. The reagents supplied have helped to identify the role of the "T" and "t" antigens in tumors of owl monkeys.

Proposed Course of the Project: This contract will be continued for another year.

Publications: None

CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal Year 1980

Microbiological Associates (NO1-NS-9-2324)

Title: Development and Delivery of Antigen, Antisera and Viral Diagnostic Reagents.

Contractor's Project Director: Dr. Gabriel A. Costellano.

Current Funding: \$478,242.00

Objectives: This is a service contract to provide reagents for the Collaborative Perinatal Research and JC papovavirus studies.

Major Findings: A large number of high quality viral diagnostic reagents have been provided. These include antigens for Herpes viruses types I and II, Cytomeglovirus, Measles, Rubella, Influenza and Coxsackie A and B. These antigens are used in an attempt to identify the etiology of perinatal infection. Enzyme-linked-immunosorbent (ELISA) tests have been developed for herpes, cytomeglovirus and measles. Preliminary studies on the efficiency of these tests in attempting to determine the etiology of perinatal infections will be made. Reagents for ELISA and hemagglutination tests for the JC virus are being developed.

Significance to the NINCDS Program and Biomedical Research: This contract provides to the Collaborative Perinatal Research Projects consistent reagents which are made under similar protocols with the same cells and strains of viruses. This allows us to test these sera for antibodies with viruses that were prevalent in 1964 -1970. Using similar production techniques, data obtained several years ago can be combined with current data. To date, over 80 publications have resulted from analyses of data from these studies. Many of the reports help establish the frequency of disease, the disease syndrome that develops and provides information on which to base rational therapeutic and preventative measures.

Proposed Course: The contract will be continued for the next year.

Publications: None

CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal Year 1980

Microbiological Associates: (NO1-NS-9-2318)

TITLE: Preparation and Delivery of Special Tissue Culture Cells, Media and Immunological Reagents.

Contractor's Project Director: Norma Parker

Current Level of Funding: \$99,500.00

Objectives: This is a service contract to provide special tissue culture cells, media and immunological reagents for use by the Branch.

Major Findings: A large lot of pretested fetal bovine serum was obtained for use in cellular immunity studies. This lot of sera was non-stimulated to human lymphocytes. Antigens for use in the various types of cell immunity studies was grown in cells produced with this lot of fetal calf serum in order to reduce non-specific cell stimulation. Large lots of pretested microeliza plates have been obtained. Several large lots of high quality alkaline phosphatase labeled anti-human IgG or IgM have been produced which are significant to NINCDS programs and biomedical research.

Production of antigens for cell immunity studies in pretested media and use of that serum in the test itself reduces the nonspecific reactions. This allows us to determine more accurately the specific reaction. Use of specialized equipment and the knowledge of highly qualified individuals on this contract allows us to be far more flexible in purchase of equipment and hiring of personnel. Thus this contract permits us to obtain good reagents at a reasonable price and to maintain a high commitment to research on neurological disease.

Proposed Course of the Project: The contract will be continued for another year.

Publications: None

CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal Year 1980

Meloy Laboratories, Inc.: (NO1-NS-7-2375)

TITLE: Isolated Housing and Care of Animals Used in Several Studies of Infectious Diseases.

Contractor's Project Director: Dr. David L. Sly

Current Annual Level: \$303,905.00

Objectives: To provide isolated housing and care of a colony of nonhuman primates consisting of several genera - example: Owls Aotus trivirgatus, squirrels Saimiri sciureus, rhesus Macaca mulatta, patas Erythrocebus patas, cynomolgus Macaca fascicularis. To provide housing and care for rodents, rabbits, guinea pigs and mice as required. The animals on experimental studies are monitored daily and biological specimens are collected as directed by written protocols.

Major Findings: A) Animals housed under this contract have been used in JC (polyoma virus) studies of which several owl monkeys have developed neurological tumors 16-24 months following intracranial inoculations.

B) Rhesus monkeys held under this contract were inoculated at 50 and 80 days of gestation with Rhesus cytomegalovirus. Some offspring exhibited hydrocephalus and cerebral calcification with microgyric following intracranial and intraamniotic inoculations.

Significance to the NINCDS Program and Biomedical Research: The goal of the NINCDS is to carry out planned, directed, research programs concerned with the diseases which damage the human nervous system. This contract provides the backup source in housing and monitoring laboratory animal models to study perinatal and neurological diseases.

Proposed Course of the Project: This contract will be continued for the following year.

Publications: None. Listed in each area of study in Experimental Pathology Section.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-00402-24-ID																					
PERIOD COVERED October 1, 1979 to September 30, 1980																							
TITLE OF PROJECT (80 characters or less) Perinatal Infections Causing Damage to the Child - Collaborative Perinatal Project																							
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																							
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COOPERATING UNITS (if any) Johns Hopkins University Univ. of CA, Los Angeles and Kaiser Hospital George Washington University Medical School; OB & FS, OD, NINCDS																							
LAB/BRANCH Infectious Diseases Branch																							
SECTION Immunochemistry and Clinical Investigations																							
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																							
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SUMMARY OF WORK (200 words or less - underline keywords) <p> The purpose of this study is to determine insofar as possible the role of <u>perinatal infections</u> in the production of fetal damage. To accomplish this, <u>clinical data</u> and a large number of serial serum specimens have been obtained from the 58,000 women and their children in the <u>Collaborative Perinatal Project</u>. Now that the project is <u>complete</u>, it is possible to study perinatal infections with three main approaches: 1) <u>clinical infections</u>; 2) <u>subclinical infections</u> detected <u>serologically</u> using abnormals and matched controls; and 3) <u>high risk</u> children with <u>elevated IgM</u> levels. Special investigation included the <u>epidemiology</u> of infections and the frequency of congenital <u>toxoplasmosis</u>. <u>Cooperating units</u> work with the Infectious Diseases Branch to study newborns in high risk nurseries. <u>Serum, IgM</u> volumes, plus clinical findings are being used to identify infected infants at risk for <u>perinatal damage</u>. Specific tests are then <u>developed</u> and <u>applied</u> for identification of the infection. Preliminary data indicates that urinary tract infection during pregnancy were found to increase the risk for abortion, stillbirths, neonatal deaths and prematurity. </p>																							

Project Description:

Objectives: The purposes of this study is to determine insofar as possible the role of infections and immunity in the production of abnormal pregnancy outcomes. To accomplish this, 12 collaborating institutions in the Perinatal Research Study plus two special cooperating groups in separate studies obtained specimens of blood and tissue throughout pregnancy, at delivery, post partum, and at set intervals thereafter. These sera are being tested to determine the antibody responses of the patients during pregnancy and post partum and then to relate this serological information to the clinical data for the pregnancy and child. In addition, serum specimens from the children were obtained at one-year-of-age from 10,000 study pregnancies. Sera, throat swabs and urine specimens were also obtained from approximately 5,000 pregnancies. Placental specimens were obtained from 2,500 pregnancies. In special cases when congenital infection is suspected on the basis of clinical or laboratory findings, throat swabs and blood specimens were obtained from the children. Immuno-globulin determinations were performed with the cord blood specimens from the children and specific antibody determinations are also being made with these specimens.

Methods Employed: To accomplish this program, blood specimens were obtained from pregnant women at set intervals throughout pregnancy and post partum. Completeness of the sets of sera is determined at the Serum Center of the Infectious Diseases Branch. Data for the 58,000 patients in the Collaborative Perinatal Research Study show that specimens are available from 94.2% of the patients. An average of five blood specimens is available for each patient. Each specimen consists of four vials with 3 ml of serum in each. For this study then, there are approximately 300,000 serum specimens and almost a million and a half vials of sera. There are an additional 5,000 patients studied to date at the Kaiser Hospital in Los Angeles and approximately 3,000 under study at the Johns Hopkins Medical School in Baltimore, Maryland. All specimens are stored at -20°C until tested and complete filing record concerning basic patient information and the status of the serum available is maintained through a computer system by the Serum Center of the Branch.

In addition to the serum specimens, serial urine and throat specimens were also obtained on a large majority of the patients in the two special studies. These are being studied for direct virus isolation along with swabs obtained from the children at the time of birth.

To date, approximately 80 publications have resulted from the analysis of the data from these studies. The serological method most frequently employed is the complement fixation (CF) test with the use of viral antigens. The test is very versatile and can be performed rapidly and provides broad coverage for a great many of the more than 130 viruses which are known to be of importance to man. Antigens were prepared for most of these viruses and tests of specificity were conducted with animal sera. In addition to the CF method, hemagglutination inhibition (HI) tests are used for many viral serological determinations. When greater specificity is needed, enzyme-linked immunosorbent (ELISA) neutralization methods are employed. Indirect fluorescence is also

used for some of the studies. Virus isolation is conducted with tissue culture or inoculation of experimental animals.

All tests are reproduced completely and a minimum of 95% agreement within 2-fold variation is required. All sera showing significant changes in antibody, together with any sera which did not reproduce are tested the third time. We are now completing the study of reported viral, bacterial and protozoal infections in pregnant women in the study. Serological tests are used to document these reports. The data is then correlated with the pediatric findings. Approximately 2,500 cases of reported viral infections, 3,000 bacterial infections and several hundred protozoal infections are under investigation. Clinical data is being abstracted, serological tests are being performed in order to document these infections. There are also approximately 1,200 patients identified with a positive serology for syphilis. These are being studied in detail.

A second approach involves a large scale study designed to investigate infection and immunity in relation to 8,000 abnormal children in the study and 8,000 matched controls. The print-out of abnormal patients has been obtained from the Collaborative Perinatal Research Study and this is being reviewed in detail by nurses and physicians from the IDB for more complete information.

From study records, the specific abnormalities under study include abortions, stillbirths, cataracts, congenital heart disease, neonatal deaths, low birth-weight (1,000-1,500, 1,500-2,000 grams), IQ below 50-69, enlarged liver, malformations, retarded gross motor development, retarded fine motor development, hearing deficit in both ears, visual impairment, cranial or peripheral nerve damage, cerebral palsy, delayed motor development, hypotonia with poor deep tendon reflexes, nonfebrile seizures, dyskinesia and ataxia, hearing deficit in one ear and elevated bilirubin. The specimens from the mothers of these children and from the children themselves along with carefully matched controls are being studied for antibody to 11 antigens. These antigens include Influenza A, rubeola, rubella, mumps, Coxsackie B₃, Coxsackie B₄, Varicella Zoster (VZ), toxoplasmosis, cytomegalovirus (CMV), Herpes Simplex type I and II. All of these agents are known or suspected to be responsible for damage in the perinatal period. All laboratory work is being performed under code. The data is being analyzed by Dr. Ellenberg. A second phase of this study will involve four additional antigens.

The third approach is to identify the children with elevated IgM levels in the newborn period and then correlate these findings with pregnancy outcome, clinical performance of the child and specific serological tests for IgM antibody. Almost 32,000 cord sera have been tested for IgM antibody and approximately 2,000 show elevated levels. These are now being studied in detail.

Major Findings:

The problem of the reproducibility of serological antibody tests for rubella and other viruses constitutes a major problem in clinical diagnosis of these infections. ELISA tests for virus antibodies provide an alternate method for comparison titrations.

Data for 28 categories of abnormalities has been tabulated from the collaborative Perinatal Project. The analysis of this information now permits us to determine the risk associated with a variety of infections during pregnancy.

Significance of the Program to the Institute: The use of new serological techniques for a large group of new viruses provides an opportunity to investigate the disease caused by viruses which are either difficult to isolate or resistant to evaluation because the clinical effects are delayed until a long time after the infection has subsided. In addition, the availability of new immunologic techniques provides the unique opportunity to detect immunologic deficits and to determine the presence of intrauterine infections on the basis of immunologic response. This data can then be correlated and analyzed as in relation to the possible causes of birth defects. The application of this type of analysis has provided valuable information on infections in relation to abnormal pregnancy outcomes and is constantly giving us new insights into the causes of damage to the central nervous system and possible means of prevention of this and other damage to the developing fetus and newborn.

Proposed Course of the Project: The combined immunologic virologic program will continue during the next year. During that time we will complete the tests for the first two phases of the serological studies. Phase three testing will then be initiated using four new antigens.

The three approaches which are being emphasized include:

1. Publication of the correlation of clinically reported infections in pregnancy with serological findings for the pregnancy, immunologic determinations and pregnancy outcome. These studies should be reported for the most part in the next fiscal year.

2. A special commitment to perform serological tests on 8,000 abnormal pregnancies and 8,000 matched controls using 11 antigens. The abnormal children have been identified and the laboratory is now approximately 80% of the way through the testing. Data analysis is being completed for the first 26 abnormal outcome categories.

3. Special test of IgM levels from 32,000 cord sera from children in the Collaborative Perinatal Research Study and in the cooperative studies. This work provides an index for identifying children with possible congenital infections so that more specific testing can then proceed. These investigations are being tested for specific antibody.

Additional studies of high risk groups will be conducted for infections with cytomegaloviruses and herpesviruses.

Publications:

Johnson, L.D., Fuccillo, D.A., Stalder, H., Oxman, M.A., Fraser, C.E.O., Madden, D.L.: Comparison of indirect hemagglutination and indirect immunofluorescence tests with microneutralization tests for detection of type-specific herpesvirus hominis antibody. Journal of Clinical Microbiology 9: 384-390, 1979.

Sever, J.L., Krasny, M.A., Ley, A.C.: Current serologic tests for herpes I, II; cytomegaloviruses; and rubella. In H. Friedman, T.J. Linna and J.E. Prier (Eds.). Immunoserology in the Diagnosis of Infectious Diseases, University Park Press, Baltimore, 1979.

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Sever, J.L.: 1979 Kimble Methodology Award Lecture: For developmental work in miniaturization of seroepidemiological techniques - microtiter system and development of diagnostic procedures and reagents in worldwide use. The Public Health Laboratory 38: 222-224, 1980.

Sever, J.L.: Serology of viral infections: Problems in reliability. Clinical Immunology Newsletter 1: 4-5, 1980.

Sever, J.L., Larsen, J.W.Jr., Grossman, J.H.III: Handbook of Perinatal Infection. Boston, Little, Brown and Company, 1979.

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Other:	John L. Sever	Chief	IDB, IRP, NINCDS																																							
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	Aurella Krezlewicz	Microbiologist	IDB, IRP, NINCDS																																							
	Matti Iivanainen	Guest Worker	IDB, IRP, NINCDS																																							
	William London	Veterinary Director	IDB, IRP, NINCDS																																							
	Maneth Gravell	Research Microbiologist	IDB, IRP, NINCDS																																							
	William Wallen	Senior Staff Fellow	IDB, IRP, NINCDS																																							
	Vince A. Calabrese	IPA Guest Worker	IDB, IRP, NINCDS																																							
	Lilly Jacobson	IPA Guest Worker	IDB, IRP, NINCDS																																							
COOPERATING UNITS (if any) University of California, Los Angeles Electronucleonics, Inc. Microbiological Associates, Inc.																																										
LAB/BRANCH Infectious Diseases Branch																																										
SECTION Immunochemistry and Clinical Investigations																																										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																																										
TOTAL MANYEARS: 5	PROFESSIONAL: 3	OTHER: 2																																								
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																																										
SUMMARY OF WRK (200 words or less - underline keywords) Continued efforts have been made to determine the etiological agents associated with <u>Multiple Sclerosis</u> . We have continued to use the <u>direct migration inhibition</u> , <u>lymphocyte cytotoxicity</u> and <u>complement mediated cytotoxic</u> tests to determine the cellular immune response of MS patients and carefully matched controls. We have developed a technique associated with <u>flow cytofluorometry</u> to measure responses in a small number of lymphocytes in an effort to determine the cellular immune response cerebrospinal fluid cells. We have continued to attempt to develop the <u>ELISA</u> technique to measure antibody against a variety of etiological agents which may be associated with multiple sclerosis or other neurological diseases. We have found that the ELISA technique is as adequate as existing serological tests to determine antibody titers but in most cases although the antibody titers are higher, the specificity of the tests are not much greater. Routine monitoring of cultures from experimental viral studies for <u>mycoplasma</u> contamination and efforts to develop new techniques to monitor tissue cultures for contamination have been continued.																																										

Project Description:

Objectives: To isolate and identify viral and non-viral antigens and/or antibodies. To utilize these antigens and/or antibodies for more specific, rapid, sensitive identification of antigens and antibodies and/or a more accurate identification of infectious agents in diseases. To define the humoral and cellular immune response of patients with neurological disease to these antigens. To determine the relationship between specific infectious agents in pregnant women and mental retardation, congenital jaundice and postnatal jaundice.

Methods Employed: Human tissue culture lines chronically infected with sub-acute sclerosing panencephalitis (SSPE) measles virus, Herpes Simplex virus types I and II (HSV-I and II), and cytomegalovirus (CMV) have been used to determine the immunological response of SSPE, and multiple sclerosis (MS) patients and matched pal controls to these antigens. The infected cells have been labeled with $^{51}\text{Chromium}$. Target cells and various concentrations of lymphocytes or serum are reacted for 18 and 24 hours and the amount of $^{51}\text{Chromium}$ specifically released is determined.

We have adapted flow cytofluorometric techniques to measure the cellular immune response of patients with multiple sclerosis. The amount of DNA and RNA in cells using the cytofluorograph has been determined by vital staining of cells in a one step method using acridine orange. The cells are stained for 8 minutes and then passed through the cytofluorograph. Analysis of the ratio of DNA to RNA present in the cell is displayed using a single phase pulse height analysis. The amount of change in DNA in specific channels is compared with the control and a stimulation index determined. The analysis takes about 2 minutes for each sample. Comparative studies using cell counts of 1×10^6 cells per milliliter to determine the amount of stimulation by cytofluorograph and conventional tridium isotope technique have been made. Currently, as few as 2×10^3 lymphocytes per culture can be analyzed by cytofluorometric techniques.

Adaptation of the enzyme-linked immunosorbent assay (ELISA) to detect antibody against several viruses associated with neurological diseases has been accomplished. The viral antigens are absorbed onto disposable plastic plates treated with serum and the unbound antibody is then washed off. Anti-human IgG conjugated to phosphatase is reacted to the serum remaining in the plate, excess washed off, substrate added and the color which develops is proportional to the amount of antibody in the unknown sera. Correlation between existing assays are very close. Efforts are being initiated to identify viral antigens using plates coated with specific antiviral antibodies.

Virus preparations and antigens used in cellular immune studies have been examined for presence of mycoplasma. Comparison of several methods for identification of mycoplasma and tissue culture are underway. Techniques to detect nonculturable strains have been adapted. The emergence of additional strains that do not grow in liquid media makes these studies extremely important.

Major Findings: Further studies on the roles of viruses in the etiology of Multiple Sclerosis have been completed. Our investigation of patients and controls from the North Central United States have been extended to populations in California and Faeroes Islands. In all populations there was a significant increase in the mean titer of measles antibody in the serum of MS patient population as compared to the control population. There was no increase in the serum titer of other virus tested as cytomegalovirus, herpesvirus types I and II or vaccine virus in the MS population as compared to the control population. In the populations studied when carefully matched patients as to age, sex, ethnic background and place of residence were studied, there were no differences in frequency of the HLA genotype between the MS and control patients. No differences in the cellular immune responses of MS patients or matched controls were detected. In the California study, the MS patients with DW2 antigens had a different humoral and cellular immune response to measles virus than those without. In the north central population, this difference was not evident. Further studies to clarify these differences are underway.

Utilizing the antibody survey techniques, efforts have been made to determine the etiology of Parkinson, Amyotrophic Lateral Sclerosis, and late onset postpoliomyelitis progressive muscular atrophy. There was no significant serological evidence to suggest a significant association with a persistent or post virus infection.

Studies completed indicate that the cellular immune responses 2×10^5 cells culture can be readily detected with the cytofluorograph. The response of cells at 3 days is similar to that observed using conventional techniques which utilize 5×10^5 cells/cultures. The amount of phytohemagglutination time and volume being similar eliminates the effect of secondary stimulation. Preliminary observation indicates that the cytofluorograph can measure the cellular immune response as early as 24 hours. Studies have been initiated to determine the cellular immune response of cells produced within the central nervous system.

Measurement of antibody against several common viruses in the serum and cerebral spinal fluid is possible utilizing the ELISA technique. A comparison of the serum/cerebral spinal fluid ratio indicates that log ratios under 2 were consistent with CNS in suite product of antibody. Presence of in suite antibody production was also related to oligoclonal antibody production.

A micromethod to detect oligoclonal IgG from 50 μ l of unconcentrated cerebral spinal fluid was developed using polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS-PAGE). This technique is as sensitive as the agarose electrophoresis technique which requires up to 5 ml of cerebral fluid which must be concentrated 50-100 times. The small volume of unconcentrated CSF required enhances the usefulness of the test. The addition of enzymed labeled antibody to the developed PAGE strip has demonstrated that the band is indeed gamma globulin. Studies utilizing these strips are being undertaken in an effort to determine the specificity of the antibody.

Progressive myoclonus epilepsy has been differentiated into several groups based upon (1) the presence of Lafora bodies in the brain and/or other tissues; (2) age at onset of disease; (3) presence of dementia; (4) course of the disease; (5) mode of inheritance; and (6) associated hearing loss. The PME syndrome most common in Finland is characterized by absence of Lafora bodies; onset in late childhood (from 6 to 15 years of age); and evidence of autosomal recessive inheritance. Finnish patients revealed lymphoid cell reaction and increased IgG level. Oligoclonal IgG bands were found in about 75% of the patients along with lowered serum/CSF antibody ratios. The presence of elevated antibody levels suggests intrathecal antibody synthesis was due to a non-specific immunostimulation.

Studies of tissue culture cell levels, seed viruses and media for mycoplasma contamination continues. Continued monitoring of common reagents used by the Infectious Diseases Branch is necessary to prevent these contaminating agents from producing artifacts in the data. Efforts to develop new and more rapid techniques for identifying these agents continue.

Significance of the Program to the Institute: The development of more specific antigens or antibodies which measure more accurately the immunological status of an individual is needed. Highly specific antigens or antibodies may help identify the biological differences between pathogenic and nonpathogenic strains of these organisms and identify the etiology of obscure diseases.

Proposed Course of the Project: Further studies will be done to identify the antigens associated with the measles and rubella, HSV-1 and II and CMV. Cellular and humoral immune studies are being expanded in an effort to detect small amounts of antigen on intact cells and immunological response differences which may account for neurological diseases.

Publications:

Visscher, B. R., Myers, L. W., Ellison, G. W., Malmgren, R. M., Detels, R., Lucia, M. V., Madden, D. L., Sever, J. L., Park, M. S., and Coulson, A. H.: HLA types and immunity in multiple sclerosis. Neurology 29: 1561-1565, 1979.

Kurent, J. E., Brooks, B. R., Madden, D. L., Sever, J. L., and Engel, W. K., CSF viral antibodies: evaluation in amyotrophic lateral sclerosis and late onset postpoliomyelitis progressive muscular atrophy. Arch Neurol 36: 269-273, 1979.

Elizan, T. S., Madden, D. L., Noble, G. R., Herrmann, K. L., Gardner, J., Schwartz, J., Smith, Jr., H., Sever, J. L., and Yahr, M. D.,: Viral antibodies in serum and CSF of parkinsonian patients and controls. Arch Neurol 36: 529-534, 1979.

Madden, D. L., Wallen, W. C., and Sever, J. L.,: Cellular immunology in multiple sclerosis response to several viruses. Humoral Immunity in Neurological Diseases. 1979.

Iivanainen, M., Leinikki, P., Taskinen, E., and Shekarchi, I.,: Oligoclonal IgG and virus antibodies in cerebrospinal fluid in progressive myoclonus epilepsy. Transactions of the American Neurological Association 103, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER <div style="text-align: right;">Z01-NS-02038-08-ID</div>
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Combined Clinical, Viral and Immunological Investigations of Acute and Chronic Diseases of the Central Nervous System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	John L. Sever Sidney A. Houff	Chief Clinical Associate
		IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	David L. Madden Maneth Gravell Monique Dubois-Dalcq Anita C. Ley	Veterinary Director Research Microbiologist Research Microbiologist Microbiologist
		IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any) University of Vermont VA Hospital, Washington, D.C. Georgetown University Medical School, Washington, D.C. Children's Hospital, Washington, D.C.		
LAB/BRANCH Infectious Diseases Branch		
SECTION Immunochemistry and Clinical Investigations		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">4.5</div>	PROFESSIONAL: <div style="text-align: center;">1.5</div>	OTHER: <div style="text-align: center;">3.0</div>
CHECK APPROPRIATE BOX(ES)		
<input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Clinical and <u>laboratory studies</u> are conducted to determine etiology (<u>infection, immunity</u> and/or <u>genetics</u>) for chronic diseases of the central nervous system. Current studies include <u>Multiple Sclerosis</u> , <u>Progressive Multifocal Leukoencephalopathy</u> , <u>Subacute Sclerosing Panencephalitis</u> , <u>Myasthenia Gravis</u> , <u>Amyotrophic Lateral Sclerosis</u> and <u>Parkinson's Disease</u> . Combined clinical data, genetic information, HLA and MLC typing, virus serology and <u>virus isolation studies</u> are obtained for these studies. Oligoclonal IgG was found in the CSF of 90% of MS patients and about 50% of the patients with Myasthenia Gravis. Parkinson patients were tested but no unusual antibody levels or oligoclonal IgG patterns were found in their serum or cerebrospinal fluids. Previously unrecognized as a possibility the human-to-human transmission of rabies by corneal transplant was demonstrated. Donors with neurological diseases must be carefully reviewed before their tissue is used in transplantations.		

Project Description:

Objectives: Clinical and laboratory studies are being conducted on chronic infections of the central nervous system (CNS). During this year, the investigations have centered primarily on multiple sclerosis (MS), progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis (SSPE), myasthenia gravis (MG), amotrophic lateral sclerosis (ALS) and Parkinson's disease. These studies have epidemiological, serological, cellular immune, viral and therapeutic components. They involve collaboration of a number of groups throughout the United States.

Methods Employed: MS patients and tissues are obtained from a number of collaborators throughout the world. New tissue culture methods and electron microscopic techniques are used in these studies as well as genetic and cellular immune tests.

Specimens from patients with PML, SSPE, ALS, MG, Parkinson's disease, and neurological complications of Epstein-Barr virus infections as well as several types of brain tumors are being studied virologically and immunologically. Individuals immunized with measles vaccine are being tested for antibody levels and persistence of antibody.

Major Findings: The CSF of about 90% of the MS patients have oligoclonal IgG. Monoclonal or oligoclonal bands appear in 50% of the myasthenia gravis patients. The occurrence of the abnormal IgG in the myasthenia gravis patients suggests that CNS involvement is more complex and more extensive than has been previously recognized. Serum and cerebrospinal fluid from patients with Parkinson, ALS and progressive muscle atrophy have been tested and no significant increase in the antibodies against a variety of viruses or oligoclonal IgG have been recognized.

Patients with neurological complications of EBV infections have been shown to have antibodies to early antigens and viral capsid antigens in the CSF. Oligoclonal bands have been shown to occur during the active stages of disease and disappear as the disease process resolves. One patient has experienced exacerbations of her illness with the reappearance of antibodies in CSF. These findings suggest the expression of EBV antigens within the central nervous system and may serve as sensitive diagnostic indicators for neurological illnesses associated with EBV infections.

The human to human transmission of human rabies by corneal transplant was demonstrated. The initiating source of infection was not identified although the occupational history of the original case suggests that wildlife exposure was possible. This study points out that donors with neurological disease must be carefully evaluated if their tissues are to be used for transplantation.

Patients with neurological complications or Epstein-Barr Virus Infections have been shown to manifest antibodies to various viral antigens in CSF as well as oligoclonal bands. One patient with encephalitis following infectious mononucleosis has been treated with Acyclovir, a new antiviral agent. The patient recovered.

Significance of the Program to the Institute: Clinical and laboratory studies of MS, PML, SSPE, MG, postinfectious polyneuritis, ALS, neurological complications of infectious mononucleosis, and Parkinson's disease permit direct investigation of the possible causes of these diseases and provide us with an opportunity to study unique "experiments" of nature which often provide very valuable insight into disease process. These studies are designed to take advantage of both the epidemiology as well as the direct laboratory approaches to the problems of acute and chronic infections of the CNS.

Proposed Course of the Project: Additional studies in attempts to identify the etiology of Multiple Sclerosis, Myasthenia Gravis, ALS and Parkinson's Disease will be continued. Emphasis will be placed upon identifying the role of the Epstein-Barr virus as a cause of neurological disease. A treatment protocol using Acyclovir for patients with complications of EBV infections has been submitted. Continued effort to identify the relationship of PML and brain tumors are being undertaken. Attempts will be made to develop methods of rapid diagnosis for viral encephalitis using a multicenter cooperative study, which has been approved. Research to identify the cause of oligoclonal IgG bands in CSF and the cellular immune response of CNS cells is continuing.

Since the first report of human to human transmission of rabies, a second case has occurred in Paris, France. The frequency of corneal involvement in natural rabies is being investigated in mice. Methods of rapid diagnosis using fluorescent antibody techniques are being developed to aid in determining suitability of individual cases for corneal transplantation. Studies of the pathogenesis of encephalitis and atypical Guillian-Barre' Syndrome due to rabies are being pursued to determine what factor(s) favor the development of one syndrome as compared to the other.

A second case of acquired rubella panencephalitis occurred in a 14 year old male who contracted a rubella virus infection during the pandemic of 1965. Rubella antibody and oligoclonal bands were present in CSF. Cerebellar biopsy revealed loss of molecular layers, cells and vacuolization as well as loss of Purkinje's cells. Attempts to rescue rubella virus or identify virus by either electron microscopy or fluorescent antibody have been unsuccessful.

Publications:

Houff, S., Madden, D.L. and Sever, J.L.: Subacute sclerosing panencephalitis in only one of identical twins, a seven-year follow-up. Arch. Neur. 36:854-856, 1979.

Williams, Adrian, McFarland, H., Eldridge, R., Houff, S., Krebs, H. and McFarlin, D. Clinical and immunologic studies on selected twins with multiple sclerosis. General Scientific Session Morning Meeting; Neurology 29:573-574, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-01731-12-ID
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Isolation, Characterization and Diagnosis of Infectious Agents From Chronic Diseases		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Maneth Gravell Other: William T. London Amos E. Palmer Marta Monzon Olajide Agbede Rebecca S. Hamilton Otto Gutenson Blanche Curfman Robert Brown	Research Microbiologist Veterinary Director Research Veterinarian Visiting Fellow Visiting Fellow Biologist Biologist Biologist Biological Lab Technician	IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any)		
LAB/BRANCH Infectious Diseases Branch		
SECTION Neurovirology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">6.0</div>	PROFESSIONAL: <div style="text-align: center;">3.5</div>	OTHER: <div style="text-align: center;">2.5</div>
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p> <u>Patas monkeys</u> shown to be <u>asymptomatic chronic carriers</u> of <u>Simian hemorrhagic fever (SHF) virus</u> were superinfected with an acute strain of SHF virus (LVR or P-180 strains). Superinfection caused elimination of both the persistent and acute viruses, thus clearing the <u>persistent infection</u>. Although the <u>persistent</u> and <u>acute virus strains</u> are <u>antigenically related</u>, they are not identical as specific antisera do not have cross-neutralizing activity. Low titers of <u>antibody</u> were induced in patas monkeys infected with the persistent virus strain, while high titers were induced by the acute strains. These results suggest that the normal antibody response is suppressed in persistently infected patas monkeys and that this <u>suppression</u> can be reversed by super-infecting these animals with an acute strain of SHF virus. </p> <p> <u>Virion polypeptides</u> of three isolates of SHF virus were studied by slab <u>polyacrylamide gel electrophoresis</u>. All three isolates contained 5 virion polypeptides of similar molecular weight ranging from about 10 K to 50 K daltons. The largest polypeptide was a glycoprotein. </p>		

Project Description:

Objectives: To use virological, biochemical and immunological techniques to study persistent viral infections and their role in chronic neurological diseases.

Methods Employed: Virion polypeptides of three strains of SHF virus were studied by slab polyacrylamide gel electrophoresis and fluorography. The antigenic relationship of these three virus strains were compared and titers of specific antibody determined by enzyme-linked immunosorbent assay. The indicator system for this test was p-nitrophenyl phosphate and alkaline phosphatase complexed to rabbit anti-human IgG. Harvests of rhesus or patas monkey macrophages were made by peritoneal lavage and were purified by centrifugation on Ficoll-Hypaque. Peritoneal macrophages were maintained in vitro by tissue culture techniques and used for assays of SHF virus infectivity.

Major Findings: Simian hemorrhagic fever (SHF), a highly fatal disease of monkeys of the genus Macaca, is caused by a member of the Togaviridae family, tentatively subclassified as a pestivirus. The patas monkey, Erythrocebus patas, a natural host of SHF virus, has been identified as the source of virus causing epizootics in laboratory colonies of macaques. Three isolates of SHF virus with different biological properties all produce a fatal hemorrhagic disease in macaques, but have variable pathogenicity for the patas monkey. The P-248 isolate produces an asymptomatic persistent infection, the LVR isolate an asymptomatic infection of short duration, and the P-180 isolate an acute hemorrhagic disease.

Superinfection of patas monkeys persistently infected with SHF virus with an acute strain of this virus (LVR or P-180 isolate) resulted in elimination of both the persistent and acute viruses, thus clearing the persistent infection. Although the persistent and acute virus strains are antigenically related, they are not identical as specific antisera do not have cross-neutralizing activity. In fact, persistent infections can be established in virus-free animals previously infected with acute strains of virus and possessing high titers of specific antibody. Low titers of antibody were induced in patas monkeys infected with the persistent strain, while acute strains induced high titers. These results imply that immunoregulatory controls are exerted to maintain persistent SHF virus infections in the patas monkey and that these controls can be over-ridden by superinfection with acute strains.

Virion polypeptides of the P-248, p-180 and LVR isolates were studied by slab polyacrylamide gel electrophoresis. The three isolates each contained 5 virion polypeptides ranging in mol. wt. from about 10 K to 50 K daltons, the largest of which was a glycoprotein.

Significance of the Program to the Institute: A number of fatal neurological diseases are caused by persistent viral infections, including subacute sclerosing panencephalitis, progressive multifocal leukoencephalopathy, cytomegalovirus inclusion disease, rubella panencephalitis, etc. Usually, irreversible damage has occurred in patients with these diseases before their cause is determined and little hope remains to arrest fatal progression of the disease. Thus, emphasis must be placed on learning how persistent infections become established, evade elimination by host immunological defenses and cause pathological damage to the host. This is the long term goal of this project.

Proposed Course of the Project: We will continue to seek information on mechanisms of SHF virus persistence, the target cells involved in persistent and acute infections, and immunological parameters associated with persistence and the clearing of persistent infections. Work will also be continued on cytomegalovirus and Herpes simplex viruses, types 1 and 2.

Publications:

Gravell, M., London, W.T., Rodriguez, M., Palmer A.E., Hamilton, R.S. and Curfman, B.L.: Studies on Simian hemorrhagic fever virus infection of patas monkeys. I. Serology, In: Proceedings of the Symposium on the Comparative Pathology of Zoo Animals. Smithsonian Institution Press, Washington, D.C., 1980.

Ferguson, M. and Murphy M.F.: Homotypic and heterotypic interfering activity associated with measles and subacute sclerosing panencephalitis viruses. The Journal of Infectious Diseases 141:414, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-01983-09-ID																										
PERIOD COVERED October 1, 1979 to September 30, 1980																												
TITLE OF PROJECT (80 characters or less) Chronic Viral Infections																												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																												
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">William C. Wallen</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 20%;">IDB, IRP, NINCDS</td> </tr> <tr> <td rowspan="7">Other:</td> <td>David L. Madden</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>John L. Sever</td> <td>Chief</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>William T. London</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Sidney A. Houff</td> <td>Clinical Associate</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Vincent Calabrese</td> <td>Visiting Scientist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Bernard Rentier</td> <td>Visiting Scientist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Renee G. Traub</td> <td>Microbiologist</td> <td>IDB, IRP, NINCDS</td> </tr> </table>			PI:	William C. Wallen	Senior Staff Fellow	IDB, IRP, NINCDS	Other:	David L. Madden	Veterinary Director	IDB, IRP, NINCDS	John L. Sever	Chief	IDB, IRP, NINCDS	William T. London	Veterinary Director	IDB, IRP, NINCDS	Sidney A. Houff	Clinical Associate	IDB, IRP, NINCDS	Vincent Calabrese	Visiting Scientist	IDB, IRP, NINCDS	Bernard Rentier	Visiting Scientist	IDB, IRP, NINCDS	Renee G. Traub	Microbiologist	IDB, IRP, NINCDS
PI:	William C. Wallen	Senior Staff Fellow	IDB, IRP, NINCDS																									
Other:	David L. Madden	Veterinary Director	IDB, IRP, NINCDS																									
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	Bernard Rentier	Visiting Scientist	IDB, IRP, NINCDS																									
	Renee G. Traub	Microbiologist	IDB, IRP, NINCDS																									
COOPERATING UNITS (if any) Microbiological Associates, Bethesda, Maryland George Washington University Medical School, Washington, D.C. Veterans Administration Hospital, Washington, DC Georgetown University, Medical Center, Washington, DC																												
LAB/BRANCH Infectious Diseases Branch																												
SECTION Neurovirology																												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																												
<table style="width: 100%; border: none;"> <tr> <td style="width: 25%;">TOTAL MANYEARS:</td> <td style="width: 25%;">PROFESSIONAL:</td> <td style="width: 50%;">OTHER:</td> </tr> <tr> <td style="text-align: center;">1.1</td> <td style="text-align: center;">0.6</td> <td style="text-align: center;">0.5</td> </tr> </table>			TOTAL MANYEARS:	PROFESSIONAL:	OTHER:	1.1	0.6	0.5																				
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:																										
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SUMMARY OF WORK (200 words or less - underline keywords) In studies on <u>multiple sclerosis</u> , abnormal suppressor T-cells were detected to measles, rubella and occasionally to Epstein-Barr virus but not to cytomegalovirus or Herpes Simplex I virus. The response is more apparent during exacerbations but the response to measles remains aberrant even in some stable patients. The loss of suppressor T-cells to brain cell antigens, axolemma, and myelin occurs frequently with patients in exacerbation and may contribute to a possible autoimmune mechanism. Attempts to isolate an infectious agent from bone-marrow samples of MS patients proved negative. A few MS patients have CNS fluid interferon (IF) which can be detected. MS patients appear to have normal immunocompetence as measured by lymphocyte stimulation or interferon production in response to general mitogens and recall antigens in vitro during exacerbations, as well as in stable phase. Immune complexes have been detected in CSF of MS patients. The T _y lymphocytes were found to participate in as well as respond to IF as natural killer (NK) cells. In tumor cell lines from JC infected owl monkeys, the JC large "T" antigen rapidly disappears from the cells upon serial culture.																												

Project Description:

Objectives: This project investigates clinical and biological significance of viral infections in the causation of chronic neurological diseases. The varied immunologic responses to viral infections are studied to determine which parameters control an infection and which contribute to persistence of the virus and result in disease. Particular emphasis is placed on infections by Herpesviruses, Types I and II (HSV-I, HSV-II), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) and polyomaviruses (JC and BK). Cell-mediated immunity appears to play a critical role in controlling these viruses. However, the relationship of immunity and its regulation to chronic, persistent infections is not understood. Therefore, we plan to study the immune response and its regulation to these viruses during chronic and acute neurologic diseases.

Methods Employed: The principal methods employed in the study of chronic viral infections of humans include: 1) virus isolation, latent genome recovery by cocultivation, chemical or mitogenic activation; 2) virus quantitation and identification by serology, host cell cytopathogenic sensitivity spectrum and fluorescent antibody techniques; 3) large scale serological surveys of materials from selected patients with specific disease entities, using fluorescent antibody assays, enzyme-linked immunosorbent assays (ELISA) and antibody dependent lymphocytotoxicity assays; 4) cell-mediated immunity (CMI) will be detected employing the lymphocyte stimulation assay for measuring effector cell functions or antigen recognition and the direct cytotoxicity or immune interferon assays for measuring effector immune mechanisms; 5) immune regulation studies will be performed using a suppressor T-cell assay recently developed to evaluate the regulatory function during viral infections which result in neurologic disease; and 6) the role of natural killer cells and its amplifications by immune interferon and its abrogation by immune complexes will be studied to determine their contribution to pathogenesis or disease control.

Major Findings:

a. Herpesviruses - Oligoclonal IgG response directed against EBV were found in a patient with MS. Antigen (EBV) specific suppressor T-cells were found during initial exacerbations of disease.

In a study of 107 young children and juveniles, a varied clinical syndrome was associated with primary EBV infections and they often do not have characteristic infectious mononucleosis or heterophile antibody responses. A frequent neurologic sequelae was found in 7/93 (8%) of the heterophile negative EBV infections.

In a study of 63 chimps, a CMV was isolated from 9 animals upon a single isolation attempt showing that these monkeys are frequently shedding virus under normal conditions of housing these monkeys. The chimp

CMVs grew in a wide spectrum of human and primate tissues and even in rabbit kidney cells (though very poorly) in distinction from human CMV. These viruses (Ch-CMV) resembled rhesus CMV in that they do not induce F_C receptors on the surface of their target cells. Serologically, they cross react with rhesus monkey serum against natural Rh-CMV and with human serum against human CMV by immunofluorescence and are difficult to distinguish by this technique.

b. Papovaviruses - JC virus has been grown in human amnion cells and in human embryonic kidney cells. However, growth patterns appear to be slow and incomplete and these cell lines do not provide sufficient virus or antigen production to be useful at this time. To date, primary fetal glial cells are the most proficient cells for growing JC virus.

Seven tumor cell lines have been established from JC infected tumor bearing owl monkeys and three cell lines from JC infected squirrel monkeys. The large "T" antigen was present in two of the cell lines; however, it rapidly disappeared upon serial passage. The remaining tumor cells retain only small "t" antigens detected only by a radioimmune precipitation technique with serum from tumor bearing animals.

An ELISA technique has been developed to detect antibody against JC virus; however, virus antigen production for this assay remains difficult to obtain.

c. Chronic neurologic diseases - The immune response of MS patients to viral (V) associated and brain cell associated (CNS) antigens remains under active study. The patients are studied for their antibody responses to myelin, basic protein and axolemma and such viruses as measles, rubella, CMV, EBV, HSV-I, coronaviruses and Vaccinia virus. Cellular immunity to these same antigens are also studied using lymphocyte simulation assay. The immunoregulatory T-cell responses to these antigens are also tested.

We have detected immune interferon in 10/30 (33%) of active patients (during exacerbation) in cerebrospinal fluid and in 2/17 (12%) of non-MS controls. In contrast, no difference between patients and controls was found in serum levels of interferon.

Immune complexes were demonstrated to be significantly elevated in 12/28 (40%) of MS patients in exacerbation using the radioimmune Raji cell assay. No differences from controls were found using serum samples from patients in exacerbation or in stable condition.

The activity of nonspecific suppressor T-cells induced by Con A appears normal in MS patients who are stable. Those MS patients in exacerbation demonstrated wide fluctuation in their responses. MS patients differed from controls in response to measles and rubella (elevated responses) but not to HSV-I or CMV. Occasional MS suppressor T-cell responses occurred to EBV. During exacerbation, many patients lose the normal suppressor response to myelin (42%) or axolemma (25%) CNS antigens. In stable MS cases only a few had a diminished response to the CNS antigens. However, in most cases that did not have suppressor T-cells, a positive CMI (lymphocyte stimulation) response was detected to the CNS antigens.

Bone marrow samples from 7 MS patients and 5 controls were found to lack a transmissible infectious agent as had been suggested in a recent report.

d. Mechanisms of immune response - In studies involving the cytotoxic function of peripheral blood lymphocytes, we have found that T_γ cells participate in both the antibody dependent lymphocyte cytotoxic (ADLC) response and the natural killer (NK) cell response. We have shown that the spontaneous NK cell activity is primarily associated with a non-T, F_C positive lymphocyte ($T^-F_C^+$) while the interferon responding NK cell primarily belongs to the $T^+F_C^+$ lymphocyte population or T_γ cells.

Proposed Course of the Project: We propose to:

- a. continue studies of the relationship of EBV to chronic or acute neurologic diseases;
- b. continue studies on the relationship of JC virus to human disease and to determine mechanisms of immune control and to continue studies of pathogenesis of JC virus in owl monkeys;
- c. continue studies on role of immunoregulatory response in patients with MS;
- d. pursue studies on the mechanisms of ADLC and NK activity and the role of T_γ cells in this response.

Significance of Program to the Institute: Herpesviruses (HSV, CMV, EBV) and polyomaviruses often establish persistent infections with neurological manifestations. The clinical spectrum of disease associated with EBV or JC virus infections has not been completely defined. These studies are designed to examine the clinical spectrum of EBV-associated diseases of children, to describe the neurological involvement associated with these infections, and to determine whether this is an etiologic or opportunistic relationship between EBV infection and neurological abnormalities. In addition, the studies of JC virus may provide useful information regarding the spectrum of diseases which this virus can induce.

The importance of delayed hypersensitivity in several viral infections has been well documented. However, the role of CMI in chronic viral diseases with neurological complications is less well studied. These studies should help define the role of delayed hypersensitivity in herpesvirus infections particularly EBV and JC virus which are followed by neurological dysfunction.

Determination of the role of immunoregulatory responses in contributing to pathogenesis in chronic neurologic diseases is of prime interest. Our studies in this area regarding patients with MS may help determine whether the disease is an autoimmune reaction and may help in understanding the role of infectious agents in this disease.

Publications:

1. Wallen, W.C., Sever, J.L., McFarlin, D.E., McFarland, H.F.: Attempt to isolate a transmissible agent from the bone-marrow of patients with multiple sclerosis. Lancet ii, pp. 414-415, 1979.
2. Wallen, W.C., Sever, J.L. and Madden, D.L.: Cellular immunology in multiple sclerosis. III. Studies on Immune Regulatory Cells. In Humoral Immunity in Demyelinating Diseases. Karcher, D., Lowenthal, A. and Strasberg, A.D. (Eds.) NATO Series Life Sciences 24: 37-46, Plenum Press (N.Y.), 1979.
3. Levine, P.H., Wallen, W.C., Lai, P.K., Jerrells, T.R. and Fucillo, D.A.: Immunologic consideration in the selection of nonhuman primate models for studies of Epstein-Barr virus associated diseases in man. Comp. Immun. Microb. Infect. Dis. 2: 243-256, 1979.
4. Wallen, W.C., Houff, S.A., Iivanainen, M., Calabrese, V.P. and DeVries, G.H.: Suppressor cell response in multiple sclerosis. Neurology 31: 1980, (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-01984-09-ID																							
PERIOD COVERED October 1, 1979 to September 30, 1980																									
TITLE OF PROJECT (80 characters or less) Maternal Infection and Pregnancy Outcome																									
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																									
<table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 30%;">William C. Wallen</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 10%;">IDB, IRP, NINCDS</td> </tr> <tr> <td rowspan="6">Other:</td> <td>John L. Sever</td> <td>Chief</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>David L. Madden</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>William T. London</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>John H. Grossman</td> <td>Guest Worker</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Renee G. Traub</td> <td>Microbiologist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Frank J. West</td> <td>Biological Lab Technician</td> <td>IDB, IRP, NINCDS</td> </tr> </table>			PI:	William C. Wallen	Senior Staff Fellow	IDB, IRP, NINCDS	Other:	John L. Sever	Chief	IDB, IRP, NINCDS	David L. Madden	Veterinary Director	IDB, IRP, NINCDS	William T. London	Veterinary Director	IDB, IRP, NINCDS	John H. Grossman	Guest Worker	IDB, IRP, NINCDS	Renee G. Traub	Microbiologist	IDB, IRP, NINCDS	Frank J. West	Biological Lab Technician	IDB, IRP, NINCDS
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COOPERATING UNITS (if any) George Washington University Medical School, Washington, D.C.																									
LAB/BRANCH Infectious Diseases Branch																									
SECTION Neurovirology																									
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																									
TOTAL MANYEARS: <div style="text-align: center;">1.9</div>	PROFESSIONAL: <div style="text-align: center;">0.4</div>	OTHER: <div style="text-align: center;">1.5</div>																							
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																									
SUMMARY OF WORK (200 words or less - underline keywords) Nonspecific immune responses of pregnant women to general mitogens and recall antigens are not different from non-pregnant control women. However, in HSV-II infected women, virus specific cellular immunity was impaired or delayed in cases of recurrent infections. Low frequencies of antigen specific (HSV-II) responses were detected during virus shedding phases when compared to post shedding phases of recurrent infections or with shedding phase during primary infections. Antibody responses to HSV-II did not correlate with protection from recurrence of infection with HSV-II. The antibody dependent lymphocyte cytotoxicity response trailed the elevation in IHA antibody following primary infection and also after recurrences. ADLC was found to be a more sensitive assay for detecting antibody to HSV-I, although it did not predict protection from recurrence of disease. A positive antibody concentration gradient was detected in cord blood samples when compared to levels of antibody to measles virus in maternal serum.																									

Project Description:

Objectives: Infectious diseases play a significant role in causing abnormal neurological development. We are in the process of studying several viruses to determine their role in the development of birth defects and neurological diseases. Sensitive virological and immunological techniques are currently being developed and applied to the investigation of the natural course of the disease induced by these viruses during pregnancy. Particular emphasis will be placed on Herpes Simplex virus, type II (HSV-II) and cytomegalovirus (CMV) infections of pregnant women.

In addition we are studying the development of fetal immunity and its role in protection against viral infections which cause neonatal disease.

Methods Employed: HSV-II and CMV are routinely isolated employing such techniques as genome rescue by cocultivation of intact cells, cellular disruption to isolate intracellular infectious virus, as well as chemical or mitogenic activation of latent genome from infected tissues, biopsies or leukocyte populations.

Several parameters of humoral and cell-mediated immunity (CMI) are routinely employed to determine a comprehensive immunologic response pattern to a viral infection. We are currently employing indirect hemagglutination (IHA), virus neutralization, fluorescent antibody assays, ELISA and the antibody dependent lymphocyte cytotoxicity (ADLC) assay for antibody measurement to these viral antigens.

Assessment of cellular immunity include lymphocyte stimulation assay, leukocyte migration inhibition and immune interferon assays. The distribution of lymphocyte subpopulations in pregnant women and newborns will be determined employing rosetting or immunofluorescent assays for quantitation of peripheral blood T- and B-cells.

General immunocompetence of host lymphocytes was performed employing the lymphocyte stimulation assay to evaluate the proliferative response to general mitogens [phytohemagglutinin (PHA), Concanavalin A (Con A) and pokeweed mitogen (PWM)] and general recall antigens (candida, mumps and staphylococcus lysate antigens). In selected cases, the ability of the individual's lymphocytes (Fc receptor cells) to participate in the ADLC assay was measured with both autologous and known positive sera.

Major Findings: Studies regarding immunity to HSV-II infection during pregnancy have revealed:

1. Symptomatic pregnant women develop the same level of cellular immunity (lymphocyte stimulation) and humoral immunity (Indirect Hemagglutination, ADLC) to HSV II and in the same frequency as symptomatic nonpregnant women;
2. Pregnancy does not compromise general immunocompetence regarding these parameters of immunity;

3. There appears to be a delay in development of CMI in recurrent infections compared to primary infections;
4. During active shedding of virus, CMI is active only in about 40% of the cases, suggesting some inhibitory factor;
5. In primary infections, ADLC response is delayed compared to IHA antibody response.

In our studies regarding passive transfer of immunity to measles virus we have found:

1. A strong concordance between ADLC, ELISA, and hemagglutination inhibition antibody between matched maternal and cord serum samples;
2. A lack of concordance between the levels of antibody detected by these assays;
3. Significantly elevated ADLC and ELISA antibody (>10-fold) in about 9% of cord serum samples compared to matched maternal serum;
4. Very high levels of ADLC antibody (>40,000) were detected in about 15% of the cases.

A model for neonatal CMV central nervous system infection was developed utilizing fetal rhesus monkeys infected with a natural rhesus CMV isolate. We have produced a CNS infection by direct inoculation of virus at 50 and 80 days of gestation. Virus was readily isolated from brain tissue after normal delivery. Maternal antibody levels were elevated during fetal infection suggesting placental transfer of virus to the mother. This model will be further developed virologically and immunologically to examine the pathogenesis of CMV infection during fetal development and persistent CNS infection.

Significance of the Program to the Institute: These studies regarding the natural course of HSV-II and CMV infections in pregnant women and in newborns may help to determine the pathogenesis of these latent, persistent viruses. In addition, these studies may delineate mechanisms of immunological control of these viruses under normal conditions. It would be of importance to determine the contribution that immunity or immune deficits play in development of viral latency or persistence and to the subsequent neurological dysfunction that may occur later in life as a result of infections with these viruses.

Proposed Course of the Project: Studies regarding the natural history of HSV-I and II and CMV in pregnant women and its consequences to newborns will continue on a longitudinal basis. Immunological studies will be used to determine: 1) the role immunity plays in control of disease; 2) tests for prognostic evaluation using various viral-related antigens; and 3) the mechanisms of protection of the newborn and the contribution of immunity to latency in the newborn.

Nonhuman primates will be employed for more definitive studies regarding the natural course of CMV and Herpes simplex virus, as well as to study the parameters of immunity during fetal development and postnatally.

Publications: NONE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-00972-09-ID									
PERIOD COVERED October 1, 1979 to September 30, 1980											
TITLE OF PROJECT (80 characters or less) Role of Viruses and Other Microorganisms in the Perinatal Period of Experimental Animals.											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> PI: William T. London Amos E. Palmer </td> <td style="width: 33%; vertical-align: top;"> Veterinary Director Veterinary Director </td> <td style="width: 33%; vertical-align: top;"> IDB, IRP, NINCDS IDB, IRP, NINCDS </td> </tr> <tr> <td colspan="3" style="padding-top: 10px;"> Other: John L. Sever William C. Wallen Blanche L. Curfman Robert L. Brown Geneva M. Brown Frank J. West </td> </tr> <tr> <td></td> <td style="vertical-align: top;"> Chief Senior Staff Fellow Biologist Biological Lab Technician Biological Lab Technician Biological Lab Technician </td> <td style="vertical-align: top;"> IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS </td> </tr> </table>			PI: William T. London Amos E. Palmer	Veterinary Director Veterinary Director	IDB, IRP, NINCDS IDB, IRP, NINCDS	Other: John L. Sever William C. Wallen Blanche L. Curfman Robert L. Brown Geneva M. Brown Frank J. West				Chief Senior Staff Fellow Biologist Biological Lab Technician Biological Lab Technician Biological Lab Technician	IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
PI: William T. London Amos E. Palmer	Veterinary Director Veterinary Director	IDB, IRP, NINCDS IDB, IRP, NINCDS									
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COOPERATING UNITS (if any) University of Pittsburgh Presbyterian Hospital, Department of Neuropathology Pittsburgh, Pennsylvania Meloy Laboratories, Inc., Springfield, Virginia											
LAB/BRANCH Infectious Diseases Branch											
SECTION Experimental Pathology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: <div style="text-align: center;">4.8</div>	PROFESSIONAL: <div style="text-align: center;">0.8</div>	OTHER: <div style="text-align: center;">4.0</div>									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) <p>Venezuelan Equine Encephalitis (VEE) has been implicated as a human teratogen. We are studying the pathogenesis of this infection in pregnant rhesus monkeys. The vaccine strain TC-83 VEE produces porencephaly, hydrocephaly and micrencephaly when inoculated intracerebrally into 100 day fetuses. Cataracts have developed in fetuses that were delivered after 140 days gestation.</p> <p>We have developed a rhesus monkey cytomegalovirus (Rh CMV) model. This virus produces CNS abnormalities in the fetus when inoculated intraamniotically into CMV antibody positive pregnant rhesus monkeys.</p>											

Project Description:

Objectives: To study the role of viruses and other microorganisms in the perinatal period, the infection of gravid and non-gravid animals of several different species by parenteral routes with various viruses and other microorganisms to determine the effects of these agents on the animals and their fetal tissues.

Attempt to recover inoculated agents from the various animals and fetal tissues and the correlation of these re-isolations with gestational age at inoculation and dosage given. Relate these findings with gross and histopathological findings. Correlate all of this information with serological findings.

Methods Employed: An investigation of the role of viruses and other microorganisms in the perinatal period by the continual use of experimental animals, tissue culture techniques, histopathological studies and serological testing. Pregnant monkeys were inoculated by various routes and times of gestation with viruses and held in isolation chambers throughout the experiment. The animals were observed and monitored by serum samples, spinal fluid, throat swabs and tissue biopsy for evidence of disease and/or effects on fetal tissue. Pregnant animals were delivered by cesarean section so all products of conception could be saved.

Major Findings:Venezuelan Equine Encephalitis

Rhesus monkey fetuses have been inoculated intracerebrally with VEE vaccine virus at 100 days of gestation.

Fetuses are delivered sequentially every 10 days postinoculation until term (160 days). We have found that after 10 days the virus can no longer be isolated from the fetal tissues. However, the fetuses show lesions in the CNS. The lesions become progressively more severe with time until at full term the animal has severe porencephaly, hydrocephaly and micrencephaly.

All fetuses delivered after 140 days gestation show signs of bilateral cataracts. At term the cataracts are fully developed in every inoculated animal.

Rhesus Monkey Cytomegalovirus (Rh CMV)

We have been able to produce congenital infection and disease in fetal Rhesus monkeys following intrauterine inoculation with Rh CMV in sera positive mothers. At 80 days gestation, five Rhesus fetuses inoculated intracerebrally with Rh CMV became infected and virus was isolated from their tissues. At birth four of these animals showed severe hydrocephalus. Five more pregnant animals were inoculated intraamniotically at 50 days gestation. Their fetuses became infected as demonstrated by virus isolation from fetal tissues. However, only two of these newborn had hydrocephalus. Control animals did not develop congenital disease nor did CMV antibody titers rise in the mothers as it did in the mothers bearing inoculated fetuses. Rh CMV is a common viral infection of feral rhesus monkeys.

About 80 - 90% of adult animals have had the disease and many are persistent excretors of the virus. This is so similar to human CMV infections that we believe the monkey model would provide a useful tool to study this important human disease and its effects during pregnancy.

Significance of the Program to the Institute:

Research for animal models for human diseases known or suspected to cause malformations of the central nervous system should provide an insight into the pathogenesis of these anomalies. Epidemiological studies have shown that there are several viral teratogens in the human populations. These could be more thoroughly studied in animal models. Environmental agents alone or in combination with infectious agents may play a role in the development of certain types of congenital malformations. Animal models would certainly be useful in the study of these conditions.

Proposed Course of the Project

The VEE study has been completed. Further working is in progress on the fetal tissues using immunoperoxidase methods to demonstrate the presence or absence of viral antigen.

We are using the Rhesus CMV model to study the immune regulation of this disease during pregnancy. Animals from the previous study are bred again and then reinoculated with Rh CMV. All of these animals have neutralizing antibody titers to Rh CMV. We want to determine if high levels of neutralizing antibodies actually protect the fetus from congenital infection and disease with Rh CMV.

We have planned to study the effects of Toxoplasma gondii trophozoites on the patas monkey (Erythrocebus patas) fetus. This has been delayed due to a shortage of pregnant patas monkeys. If this problem should be resolved this year, a pilot study will begin in this area.

Publications

Palmer, A.E., London, W.T., Sly, D.L. and Rice, J.M.: Toxemia of Pregnancy (preeclampsia, eclampsia, hypertensive disorders of pregnancy). Spontaneous Animal Models of Human Disease, Vol. I. Ed. E.J. Andrews, B.C. Ward and N.H. Altman. Academic Press, New York, Chapter 88, 213-215, 1979.

Krous, H.F., Altshuler, G., London, W.T., Palmer, A.E., Fuccillo, D.A. and Sever, J.L.: Congenital Hydrocephalus, Model No. 163. In Handbook: Animal Models of Human Disease, Fasc. 8. Ed. T.C. Jones, D.B. Hackel and G. Migaki. Registry of Comparative Pathology, Armed Forces Institute of Pathology, Washington, D.C., 1979.

Palmer, A.E., London, W.T., Sly, D.L. and Rice, J.M.: Toxemia of Pregnancy. In Handbook: Animal Models of Human Disease, Comp. Path. Bulletin, XII, No. 1, 2-4, Feb. 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-01986-09-ID
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Inoculation of Animals with Tissue Culture Grown Materials from Patients with Chronic Neurological Diseases		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	William T. London Amos E. Palmer	Veterinary Director Veterinary Director
		IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	Sidney A. Houff John L. Sever Blanche L. Curfman Geneva M. Brown Robert L. Brown	Clinical Associate Chief Biologist Biological Lab Technician Biological Lab Technician
		IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any) Meloy Laboratories, Springfield, Virginia Microbiological Associates, Bethesda, Maryland		
LAB/BRANCH Infectious Diseases Branch		
SECTION Experimental Pathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.4	PROFESSIONAL: . 0.4	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Young cynomolgus monkeys <u>Macaque cynomolgus</u> that were inoculated intracerebrally (IC) with "Biken" strain of Subacute Sclerosing Panencephalitis (SSPE) virus were monitored. One animal has had frequent grand mal seizures for the last 12 months (one or two seizures per week). This animal exhibits measles antibody and oligoclonal bands in its sera and CSF. Several attempts have been made to infect patas monkeys <u>Erythrocebus patas</u> with human varicella (HU-ZV). All have been unsuccessful until we inoculated the virus directly into the dorsal root ganglion T7 area. At this time, the animal has developed high serum and CSF antibody titers to HU-ZV. A varicella-like virus, "Delta agent" readily infects patas monkeys and has been reported to become latent in the patas monkey. We have been able to activate a latent infection of Delta (DHV).		
40 - IDB/IRP		

Project Description:

Objectives: Develop nonhuman primate models for the study of chronic neurological diseases (other than spongiform encephalopathies).

Methods Employed: Measles antibody negative cynomolgus monkeys were inoculated intracerebrally with subacute sclerosing panencephalitis (SSPE) "Biken" strain virus and monitored for infection and clinical signs.

Laboratory reared patas monkeys that are antibody negative to Delta and HU-ZV viruses are inoculated in the dorsal root ganglion.

Major Findings:

a. SSPE studies - One of the cynomolgus monkeys developed clinical neurological signs about 30 months post intracerebral inoculation with "Biken" strain of SSPE virus. The grand mal seizures have increased in frequency and duration over the last 12 months and are now controlled by treatment with Dilantin 25mg/day. This animal has very high persisting measles antibody and oligoclonal bands in its sera and CSF.

We have been trying to develop a nonhuman primate model for human varicella zoster virus infections. We were finally successful in producing infection when we inoculated an adult patas monkey with HU-ZV into the T-7 dorsal root ganglion. This animal had been antibody negative and 60 days later had high levels of HU-ZV antibody in sera and CSF.

The "Delta agent", a varicella-like virus has been isolated from patas monkeys. This virus has been reported to be latent in patas monkeys. However, no one has reported activation of the latent infection and isolation of the Delta virus. We have demonstrated this process by supra-infecting a "Delta agent" latent patas monkey with another virus and then rescued the latent Delta virus in a sentinel patas monkey which was caged with the treated animal. Additional animals are now under study to confirm these findings.

Significance of the Program to the Institute: Chronic neurological disease represents by far the major portion of the practice of neurology in the United States. Primate models may give answers to the pathogenesis of these diseases. Pathogenic principles derived from these models may then be applied to other chronic neurological diseases.

Proposed Course of the Project:

(A) SSPE project: We will continue monitoring the animal exhibiting seizures by electroencephalography, computed tomography, cellular and humoral immunity and oligoclonal banding. Additional animals will be immunized with measles vaccine. When they have elicited a high antibody response, these animals will be inoculated intracerebrally with "Biken" strain of SSPE virus. Each animal will be observed for development of disease.

(B) Human Varicella Zoster Virus: Additional animals will be inoculated via dorsal root ganglion. After infection has occurred and high levels of antibody develop, the animals will be immunosuppressed and monitored for development of zoster-like lesions.

(C) Latent "Delta Agent" Infection: We have several animals that are latent carriers of "Delta agent" varicella-like virus. We plan to investigate the immune mechanisms related to this virus reactivation by supra-infection with another virus.

Publications:

Zook, B.C., London, W.T., DiMaggio, J.F., Rothblat, L.A., Sauer, R.M. and Sever, J.L.: Experimental lead paint poisoning in nonhuman primates II. Clinical pathologic findings and behavioral effect. J. Med. Primatol. Vol. 9 No. 5 1980.

Zook, B.C., London, W.T., Wilpizeski, C.R. and Sever, J.L.: Experimental lead paint poisoning in nonhuman primates III. Tissue lead and anatomic pathology. J. Med. Primatol. Vol. 9 No. 6 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-02136-06-ID
PERIOD COVERED October 1, 1979 to September 30, 1980 TITLE OF PROJECT (80 characters or less)		
Control of Acute Infectious Diseases in Experimental Animals Using Biologicals and Chemotherapeutic Agents		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	William T. London Veterinary Director Amos E. Palmer Veterinary Director	IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	John L. Sever Medical Director, Chief William C. Wallen Senior Staff Fellow John W. Larsen Guest Worker Blanche L. Curfman Biologist Robert L. Brown Biological Lab Technician Geneva M. Brown Biological Lab Technician Robert M. Chanock Medical Director	IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS LID, IRP, NIAID
COOPERATING UNITS (if any) LID, IRP, NIAID Meloy Laboratories, INC., Springfield, Virginia Microbiological Associates, Bethesda, Maryland		
LAB/BRANCH Infectious Diseases Branch		
SECTION Experimental Pathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.4	PROFESSIONAL: 0.4	OTHER: 2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Group B Streptococcus type III (GBS) studies - We found that an experimental polysacch- aride GBS type III vaccine did not elicit an immune response in 10 rhesus monkeys (<u>Macaca</u> <u>mulatta</u>). This presents serious problems for proposed studies using this vaccine to immunize monkeys and probably humans to GBS type III. Cytomegalovirus: We were unable to infect chimpanzees (<u>Pan troglodytes</u>) with <u>human</u> <u>cytomegalovirus</u> AD-169. We found that in a colony of 70 chimpanzees, 10 were actively shedding a cytomegalovirus (CMV) that closely resembled simian CMV. All animals tested had preexisting antibody to CMV which cross-reacted with human AD-169 CMV. Herpes encephalitis: <u>Herpes Simplex virus type I (HSV 1)</u> produces encephalitis in rhesus (<u>Macaca mulatta</u>) and cynomolgus (<u>Macaca fascicularis</u>) monkeys provided the animals are antibody negative for all herpes viruses. This gives us a model to study early diagnosis of herpes encephalitis. <u>Varicella-like Delta Agent encephalitis</u> : The Delta virus will produce encephalitis in young antibody negative patas monkeys (<u>Erythrocebus patas</u>). This model permits us to test new therapeutic agents for treatment of varicella encephalitis.		

Project Description:

Objectives: To study prophylactic and therapeutic agents for the prevention and control of infectious diseases. The testing of candidate vaccines as to their immunogenicity, communicability and safety in experimental animals.

Methods Employed: New chemotherapeutic agents which show promise are studied in appropriate experimental animals. The animals are inoculated with a known infectious agent, then a therapeutic regimen is started, using the test drug. Additional animals are prophylactically treated with the drug, then challenged with the infectious agent.

Biological agents are tested for their ability to protect animals against naturally occurring and experimentally produced infectious diseases. Newly developed vaccines will be tested in susceptible experimental animals. Vaccinated animals will be exposed to susceptible sentinel animals to determine communicability. Vaccinated animals will be challenged at appropriate times to determine the immunogenicity of the vaccine.

Major Findings:

Group B Streptococcus: The recently developed GBS type III polysaccharide vaccine was not immunogenic in 10 non-pregnant rhesus monkeys. This has prevented us from using this vaccine to immunize pregnant rhesus monkeys and later challenge them with virulent GBS.

Cytomegalovirus: We made several attempts to infect chimpanzees with human cytomegalovirus (CMV) AD-169 strain. Each time we found that the animals had preexisting antibody titers to CMV. The entire chimpanzee colony was surveyed to define the frequency of CMV infection. The results demonstrated that 10 of 70 chimpanzees shed CMV from either the throat, vagina or in the urine. All but one of these were laboratory raised animals. Biological characterization of the isolated virus was performed. The isolated virus closely resembled simian CMV and not the human virus.

Herpes Encephalitis: Using antibody negative rhesus and cynomolgus monkeys, we have produced herpes encephalitis by direct intracerebral inoculation of Herpes Simplex type I virus. The infection was fatal in the cynomolgus monkeys. The rhesus developed a focal encephalitis from which the animal recovered, but continues to have neurological deficits.

Varicella-like "Delta agent" encephalitis: This virus, the "Delta agent", has properties that closely resemble human varicella virus. Intracerebral inoculation of antibody negative patas monkeys has produced encephalitis in the animals. This model can be used to study pathogenesis, diagnosis and treatment of human varicella encephalitis.

Proposed Course of the Project:

Group B Streptococcus: To determine if high maternal antibody to GBS type III will protect the fetus when challenged with virulent organisms. We will use animals from previous studies that have high antibody titers to GBS. These animals will be rebred and then inoculated intraamniotically with GBS 24 hours prior to delivery. Modified immune serum (human) commercially produced has been suggested as part of the therapy in the critically ill newborn infant. This material has shown promise in rats and

mice. The rhesus monkey model will be used to determine the efficacy of this product in a nonhuman primate. Antibody negative mothers will be inoculated with GBS intraamniotically 24 hours prior to cesarean delivery. One half of newborns will receive intravenous treatment with the modified immune serum at birth and 72 hours. The remaining untreated newborns will serve as controls.

Herpes Encephalitis: We hope to induce persistent HSV infection of the trigeminal ganglion, by inoculation on the cornea prepared by abrading the surface of the eye. The "reactivation" of virus from the ganglion will be attempted by stress, i.e. steroids and epinephrine to produce encephalitis. In vivo, HSV antibody tagged with Iodine 125 will be used to radiographically define lesions containing herpes antigen using brain scanning with computerized tomography. We plan to evaluate a number of antiviral agents using this monkey model.

Varicella encephalitis: While we are currently studying the Delta herpes virus - patas monkey system as a model for severe complications arising from varicella zoster virus (VZV) induced disease in humans, we also are trying to ascertain whether the same DHV - patas system can be manipulated for studying the recrudescent VZV infection - "shingles". The most important and severe form of herpes zoster occurs in immuno-suppressed cancer and organ transplant patients. We would like to determine if the latent DHV can be activated to produce disease; and if so, in what manner (using immunosuppression and/or pharmacologically induced stress).

Significance of the Program to the Institute: Experimental animal studies permit the study of human diseases, their prevention and treatment with chemotherapeutic agents and biological products. Such studies provide information of efficacy, safety and side effects of these products. Information gained from experimental animal studies provides the bridge to the implementation of clinical studies in man.

Publications:

Prince, G.A., Suffin, S.C., Prevar, D.A., Camargo, E., Sly, D.L., London, W.T. and Chanock, R.M.: Respiratory syncytial virus infection in owl monkeys: Viral shedding, immunological response, and associated illness caused by wild-type virus and two temperature sensitive mutants. Infection and Immunity, 26, No. 3, 1009-1013, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-02271-04-ID
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Papovaviruses in Non-human Primates		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	William T. London Sidney A. Houff	Veterinary Director Clinical Associate IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	William C. Wallen John L. Sever Kenneth G. Rieth Giovanni Di Chiro Paul E. McKeever Carlo Buonomo Blanche L. Curfman Robert L. Brown	Senior Staff Fellow Chief Staff Radiologist Chief Medical Officer Biological Aid Biologist Biological Lab. Technician IDB, IRP, NINCDS IDB, IRP, NINCDS DR, CC NCT, SNB, NINCDS SNB, NINCDS NCT, SNB, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any) University of Wisconsin Medical School, Departments of Medical Microbiology and Pathology, Madison, Wisconsin; DR, CC, NIH; SNB, NINCDS Meloy Laboratories, Inc., Springfield, Virginia		
LAB/BRANCH Infectious Diseases Branch		
SECTION Experimental Pathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.4	PROFESSIONAL: 0.4	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Ninety two owl monkeys <u>Aotus trivirgatus</u> and 15 squirrel monkeys <u>Saimiri sciureus</u> that were inoculated 22 - 26 months ago with JC virus, a human polyomavirus or control material have been maintained and monitored this year. To date 3 owl monkeys and one squirrel monkey have developed intracerebral neoplasms.		

Project Description:

Objectives: To study the pathogenesis of papovavirus induced tumors and disease in non-human primates.

Three serologically distinct papovaviruses have been isolated from humans. The JC and SV-40-PML strains have been isolated from patients with Progressive Multifocal Leukoencephalopathy (PML). BK virus has been isolated from the urine of renal transplant patients and at least one normal child.

Inoculation of JC, SV40-PML and BK viruses in hamsters, rabbits, rats, mice and bovines has resulted in tumors of various types. We have reported that JC virus inoculated by multiple routes produce astrocytomas in 2 of 4 adult owl monkeys.

We presently are confirming our original findings using a larger sample size, determining if the primary tumors from the original two owl monkeys can be transmitted to other owl monkeys, and investigating immune modulation of owl monkeys inoculated with JC virus to determine if this results in lesions resembling PML. Finally, is a closely related new world non-human primate, the squirrel monkey Saimiri sciureus more susceptible to JC infection than the owl monkey?

Methods Employed:

Twenty adult feral Colombian owl monkeys (Aotus trivirgatus) were inoculated intravenously (IV) and intracerebrally (IC) with JC virus in attempts to confirm the original studies. Several owl monkeys were inoculated using a single variable route. This series included: a) intracerebral; b) intraperitoneal; c) intravenous; and d) inhalation. Another group of owl monkeys has been inoculated with primary tumor cells from the 2 original owl monkey gliomas.

Several owls have been immunosuppressed and then inoculated with JC virus attempting to produce PML-like lesions. Twenty-two monkeys were used as controls. A total of 92 animals were used in these studies.

Ten squirrel monkeys were inoculated with JC and 5 were inoculated with control material.

Major Findings:

Three owl monkeys inoculated via multiple routes developed glioblastomas or Grade III astrocytomas 16 - 19 months post inoculation. One squirrel monkey developed a glioblastoma 15 months following intracerebral inoculation of JC virus. All tumors were located prior to symptoms in the animals by use of computed tomography (CT). This is very important because we now have a method for early diagnosis and can intervene with treatment of tumors before terminal signs and symptoms develop.

Significance of the Program to the Institute: Demyelinating diseases are a major cause of neurological disability in the United States. Multiple Sclerosis, Schilder's disease, Devic's syndrome, post vaccination encephalomyelitis as well as PML are all illnesses characterized by loss of or defective myelin. The study of a known viral induced demyelinating illness will hopefully give us the basic knowledge which is needed to

understand the pathogenesis and etiology of the major white matter diseases of man. Brain tumors account for a relatively high proportion of all neurological disease. Gliomas are the most frequent tumors seen in man. JC virus has been shown to induce gliomas in primates. This is the first animal model of gliomas which will allow studies of pathogenesis, diagnostic techniques, and therapeutic trials applicable to human disease.

Proposed Course of the Project: Continue to monitor the owl and squirrel monkeys on this study. Each monkey's general physical condition is closely followed using monthly weights, sera, antibody titers to JC virus and quarterly hemograms as parameters. If weight loss is observed, CT scans will be done immediately to locate a possible tumor.

If animals develop tumors they will be killed and the following studies done:

- a. Attempts to rescue the virus from tumor using co-cultivation techniques.
- b. Hybridization between JC virus DNA and DNA extracted from tumor cells will be done to delineate portions of JC virus DNA present in the tumor genome. These attempts may define which portions of JC virus are required for tumor induction in the non-human primate.
- c. Neuropathological examination of tumor tissue will be done using both light and EM microscopy. Similarities and dissimilarities to human tumors will be emphasized.
- d. As additional animals develop tumors, biopsies will be taken. Using in-vitro cytotoxicity tests, the chemotherapy of choice will be determined and administered to the animal with tumors.

Publications

Rieth, K.G., Di Chiro, G., London, W.T., Sever, J.L., Houff, S.A., Kornblith, P.L., McKeever, P.E., Buonomo, C., Padgett, B.L. and Walker, D.L.: Experimental Glioma in Primates: A Computed Tomography Model. J. of Computer Assisted Tomography, 4(3):285-290, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-NS-02034-08-ID
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Electron Microscopic Studies of Viruses of the Nervous System and Demyelination		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Monique Dubois-Dalcq Research Microbiologist IDB, IRP, NINCDS Other: Dr. B. Rentier Visiting Associate IDB, IRP, NINCDS Dr. B. Burge Senior Microbiologist IDB, IRP, NINCDS Anne Claysmith Biological Lab. Technician IDB, IRP, NINCDS Ray Rusten Biological Lab. Technician IDB, IRP, NINCDS Annik Baron Biological Lab. Technician IDB, IRP, NINCDS Collaborators: Dr. B. Trapp Postdoctoral Fellow LNNS, IRP, NINCDS Dr. W. Bellini Senior Staff Fellow NIB, IRP, NINCDS		
COOPERATING UNITS (if any) Dr. M. Haspel, Dental Institute, NIH; LNNS, NINCDS: NIB, NINCDS Dr. Aguayo, Montreal General Hospital, Montreal, Quebec Drs. Knobler, Oldstone and Lampert, Scripps Clinic and UCSD, La Jolla, Calif.		
LAB/BRANCH Infectious Diseases Branch		
SECTION Electron Microscopy		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 5.7	PROFESSIONAL: 2.7	OTHER: 3.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) New techniques have been developed for identification of neurons in culture and for localization of viral antigen on vibratome sections of the nervous system. The maturation of a demyelinating mutant of mouse hepatitis virus in cultured nerve cells was found to be different from that of the wild type virus. Similarly, this mutant has a reduced tropism for neurons <u>in vivo</u> but specifically infects oligodendrocytes and causes progressive demyelination in mice. A chronic measles infection of spinal cord neurons has been characterized and all viral proteins are expressed but there is a lack of incorporation of capsids into the viral bud, which results in non-infectious virions. Rat Schwann cells, the myelin-forming cells of the peripheral nervous system, have been cultured, stimulated to mitosis, grown in large quantities and studied after multiple passages. They have preserved their characteristic motility, consisting of alternation of migration and undulation. Preliminary results seem to indicate that they are still able to myelinate. Schwann cells are susceptible to infection with rhabdovirus but an unusual pattern of viral expression was observed in these cells.		

Project Description:

Objectives: To study with combined morphological and immunological techniques: 1) mechanisms of acute and chronic infection of dissociated nerve cells with a variety of viruses known to produce diseases of the central nervous system (CNS) in animals and/or man; 2) interaction between antibodies and lymphocytes with the surface of cells infected with these viruses; and 3) brain tissue from animals and patients with acute and chronic viral infection and/or demyelinating diseases; 4) the behavior and myelinating properties of cultured Schwann cells and oligodendrocytes.

Methods Employed: Tissue culture cell lines of various origin as well as mouse CNS cultures are investigated after viral infection, with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), with or without labeling of viral antigen using Protein A peroxidase. New techniques have been developed for freeze fracturing monolayers of infected cells and for combining procedures with etching and rotary shadowing. Normal and viral infected cells are studied by video-intensification microscopy.

Major Findings1. Development and use of new techniques and surface markers.

a. The surface of cultured cells infected with neurotropic viruses such as measles, can now be visualized at high resolution using field emission SEM or rapid freezing (J. Cell Biol. 81:275-300, 1979). Brief treatment of measles-infected monkey kidney cells with anti-measles antibodies induced the formation of antigen-antibody complexes which later became redistributed in patches, caps or rings on giant cells. As detected by protein A peroxidase, SEM examination of the labeled cells after critical point drying revealed granules of peroxidase reaction product clustered on small and large buds. The label had cleared from the remaining cell surface which showed only particles also present in uninfected cells. Measles virus glycoproteins can also be visualized directly without labeling, following another procedure; glutaraldehyde-fixed cells were treated with 10% methanol, rapidly frozen, freeze-fractured, deep-etched at -104°C and replicated by rotary shadowing of the platinum. On the cell surface, viral subunits, probably corresponding to oligopeptides, were organized in typical ridges (fig 3). In other areas, both surface and protoplasmic membranes were exposed while etching revealed cytoskeleton in fractured villi (fig 3). Thus, startling improvement in resolution obtained with some SEM systems and rapid freezing with replication, now allow detailed study of viral protein organization on the surface of cells infected with enveloped RNA viruses.

b. The selective interaction of phosphoryl-choline binding myeloma proteins with mouse neurons has been described for the first time using an immunofluorescence technique (Dr. Hooghe et al.).

2. Studies of the behavior and structure of rat primary and secondary Schwann cells in vitro.

Schwann (S) cells have a remarkable remyelinating potential in PNS and CNS. Therefore, it is of great interest that numerous primary (I) and secondary (II) rat S cells can be obtained by dissociation of neonatal sciatic nerve and subculture in the presence of mitotic factors, cholera toxin and pituitary extract (Brain Research 165: 105, 1979). Freshly dissociated I S cells contain myelin fragments and specifically, immunostain for the major protein of PNS myelin (Po). During the first days in vitro these myelin components progressively disappear in cells grown in medium containing fetal calf serum and high glucose but not in synthetic medium. Video-intensification microscopy revealed that I S cells form groups and have typical rhythmic pulsations and side-to-side movements. Processes of bipolar S cells extend and retract, sometimes leading to cell migration. In contrast, II S cells are multipolar, show less pulsation and migration but extend longer exploratory processes than I S cells. They become flat and almost confluent before intense mitotic activity occurs. Withdrawal of growth factors results in partial return to bipolar shape. I and II S cells show numerous intermediate filaments and no basement membrane. I cultures contain $\pm 5\%$ Thy 1.1 positive fibroblasts which show distinct variable shape and edge ruffling. II cultures are free of fibroblasts after Thy 1.1 immune-mediated killing. The motility pattern of I S cells was compared to that of II S cells after intense mitotic stimulation by cholera toxin and a pituitary extract (Raff et al., Cell 15:813, 1978). Continuous recording by time-lapse video intensification microscopy (VIM) allowed analysis of various S cells movements. In I S cells, slow rhythmic undulation episodes were observed as described (Pomerat, Science 130:1759; Forman et al., Soc. Neurosci. Abstr. 5, 1955, 1979) and lasted 2.3 ± 0.2 min. In spite of the extreme variability of the interval between these episodes, the total number of episodes per day remained rigorously identical for different cells (166.3 ± 0.2). Short and long intervals occurred at random. Cycles, consisting of an undulation episode followed by a resting interval, had mean durations of 8.6 ± 4.1 min. and a sharp peak of occurrence at 6 min., with exponential distribution of the longer periods, indicating a non-random event. Migratory events often alternate with undulatory episodes. Migration speed was 135 ± 50 μ per hour and the path of migration was often oriented along a constant axis for every cell, in contrast with the random migratory path of occasional fibroblasts observed in I cultures.

During mitotic stimulation, subcultured S cells acquired a "fried egg" shape and both their undulatory and migratory activities were dramatically reduced. Undulation was replaced by a pulsation of approximately the same periodicity, but occurring in only 2% of the cells. Migration was reduced to 24 ± 2 μ per hour. Six to 12 hours after removal of mitogenic factors, 80% of the S cells started pulsating twice as fast for 2 to 3 days. When mitosis ceased, S cells quickly recovered their spindle shape and rhythmic undulation while their migration speed increased to 92 ± 20 μ per hour. When II S cells were

seeded over dissociated neuron cultures, they rapidly attached to neurites and showed migratory and undulating movements along these neurites. In conclusion, VIM revealed that 1) frequency of undulation episodes show a remarkable uniformity, perhaps demonstrating a genotypic basis; 2) in spite of dramatic modification of shape and behavior during mitotic stimulation, S cells subsequently recover their unique motility pattern which might be essential for their myelinating function.

The myelination ability of cultured S cells was studied in vitro and in vivo in collaboration with Drs. Trapp and Aguayo respectively. I or II S cells in large quantities were trypsinized and mixed with dissociated rat brain cells in various ratios. Cells were then allowed to rotate constantly and aggregate. Normally, rat brain cells do reaggregate and mature in three weeks. Central myelination is then observed (Trapp, et al., Brain Research, 160:117-130, 1979). Preliminary results suggest that I and II S cells mixed with rat brain cells in suspension also form aggregates which show myelination three weeks later by immunocytochemical staining for Po protein.

In another set of experiments with Dr. A. J. Aguayo in Montreal, I and II S cells were dissociated, pelleted and implanted in a vessel sutured to the proximal and distal extremities of a transected sciatic nerve in immunosuppressed mice. (Aguayo et al., Ann. N.Y. Acad. Sci., 317:512-531, 1979). It is hoped that the implanted S cells will myelinate the regenerating nerve. The mouse nerves will be examined for myelination 2 months after implantation.

3. Acute and chronic viral infections of dissociated nerve cell systems with various RNA viruses.

a. Viral expression in nerve cells often differs from that in undifferentiated cells. The replication of a prototype RNA virus, VSV, was studied in cultured rat Schwann cells, the myelin forming cells of nerves. In cultures infected with 10-40 PFU per cell, cell rounding and VSV-specific fluorescence was apparent after 2 hours of infection. Infectious virus in the medium increased from 2 through 6 hours to a peak of 20-40 PFU per cell, when cells were brightly fluorescent and often detached. Gel electrophoresis of ³⁵S methionine-labeled cell extracts showed near maximal virus specific protein synthesis by 2 hours, and the rate of incorporation dropped, at 6 hours, to less than 5% of the control rate. All virus proteins were produced in normal molar proportions. Transmission and scanning EM revealed few budding sites and no clustering of RNP strands under the membrane, in contrast to other productive cells. Thus, when compared to other cell lines, virus protein synthesis in Schwann cells is accelerated while the host cell appears to limit the production of both RNP and infectious particles.

b. The implication of measles virus as the etiological agent of several neurological diseases prompted us to attempt measles infection of dissociated neuron cultures

from mouse embryo spinal cord. Cells were infected at 10 days with a high multiplicity of plaque-purified Edmonston measles virus (MV). Two days after infection, immunofluorescence (IF) revealed MV antigens in the cell bodies of 5 to 10% of the neurons. After 7 days and up to 40 days, neither cell fusion, lysis, nor other cytopathic effects were seen while 30 to 40% of the neurons were infected as shown by IF, immunoperoxidase and hemadsorption. These markers were found on cell bodies as well as on the intricate neurite network, but not on other cell types. Supernatants from infected neuron cultures failed to infect Vero cells or other neuron cultures and did not interfere with infection of Verocells by parental virus. Electron microscopy revealed accumulation of both fuzzy and smooth nucleocapsids in neuron cytoplasm, but no capsid alignment beneath the plasma membrane. The latter was covered with numerous buds and coated with MV antigens. Most buds lacked coiled nucleocapsids and contained vesicles and microtubular structures. Polypeptide analysis of cell lysates showed synthesis of all MV proteins (Dr. W. Bellini). Preliminary data indicated that the HA and P polypeptides differed in electrophoretic mobility when compared to parental virus. In conclusion, a selective and persistent infection of mouse neurons was obtained with a non-neuroadapted strain of MV. Although all components of MV were made, incorporation of nucleocapsids into budding virions was defective and no infectious virus was produced.

- c. Study of viral expression and maturation of mouse hepatitis virus in mouse neuron cultures. Comparison between wild type (wt) and a temperature sensitive mutant (ts 8) which causes demyelination (with Drs. Haspel, Knobler, Lambert and Oldstone).

All experiments were performed at 37°C, a semi-permissive temperature. Both viruses induced giant cell formation in non-neuronal cells surrounding the neurons (glial or fibroblastic cells). Cytopathic effects were slower with ts 8 and recruitment of cells only attached by a narrow bridge to the giant cell was seen. A number of neurons displayed viral antigen late in infection with both viruses but more with wt than ts 8. Scanning and transmission electron microscopy revealed that in the middle of the giant cell, scattered virus (more with wt than ts 8) lay over the cell surface sometimes in a membrane depression (as if the virus has just been released by fusion of its vacuole with the cell membrane). At the periphery of giant cells at a later stage, numerous virions were seen budding from villi and folds and others were seen in vacuoles opening at the cell surface. Virtually no virus was found on the neurite and neuron surfaces although some intracellular virions were detected in the neuronal cell body. Some virions were seen interacting with nerve terminal membranes and some of the synaptic vesicles contained dense particles resembling virus.

Intracellular maturation appeared to differ between wt and ts 8. In the case of wt, granules and cores of viral RNP surrounded vacuoles where the virus assembled and budded. Budding sometimes occurred in Golgi vacuoles and

occasionally, directly from the plasma membrane. With ts 8 frequent smooth membrane cisterns were seen close to very large RNP inclusions. They resembled tubulo-reticular structures and sometimes contained viruses and 30 nm tubules. Rare cellular microtubules had RNP material clustered along their length. The most striking feature of ts 8 infection was the frequent occurrence of myelin-like whorls containing cytoplasm and viral RNP or buds in their center. (Whorls contain as many as 40 pairs of membranes with an interspace of 12-20 nm). Fewer virions appeared to form as compared to wt.

In conclusion, even at a semi-permissive temperature, the maturation of the mutant in non-neuronal giant cells is not similar to that seen in the infection with wt. Of particular interest was the apparent stimulation of membrane formation by the mutant.

4. Animal studies of a virus-induced acute and chronic demyelinating disease.

Wild type (wt) mouse hepatitis virus (MHV) type 4 produces an acute fatal encephalo-myelitis in adult mice. In contrast, a temperature sensitive mutant (ts 8) rarely produces the fatal disease, yet is frequently associated with demyelination. Improved intracellular penetration by antiviral antibodies and conjugates was developed in the nervous tissue of virally infected animals. Mice were perfused through the heart with 4% paraformaldehyde and 0.5% glutaraldehyde. 10 μ vibratome sections were cut from the spinal cord and treated with saponin-albumin before incubation with rabbit anti-MHV serum and protein A coupled to fluorescein or peroxidase. Alternately, sections were frozen and thawed quickly in a glycerol-sucrose mixture. Flat sections containing peroxidase-labeled cells detected by light microscopy, were embedded in epon, circled, drilled out, and remounted for thin sectioning. The label was specifically localized in neurons, on virus budding into vacuoles and throughout the cytoplasm (fig. 1). This fixation allowed preservation of antigenicity and ultrastructure. In addition, vibratome sections can be studied in light, fluorescent, and electron microscopes, permitting correlation of pathological change with identification of virally infected cells as well as fine structural localization of viral components.

Animals inoculated with wt virus showed antigen in neurons, satellite cells and scattered oligodendrocytes. Occasionally, granules of antigen were seen in endothelial cells and the periependymal area (under EM, a virion was seen directly beneath the basal lamina). Three to five days postinoculation with wt, antigen was found in the substantia gelatinosa neurons and in various neurons of the midbrain, whereas 7 to 8 days postinoculation anterior horn neurons were more frequently labeled and occasional demyelination was seen. Some animals did not show much antigen at the cervical level but did seem to show lumbar and thoracic involvement.

EM revealed that with wt, infected neurons often had diffuse label throughout the cytoplasm. Label was located on rough ER and virions as well as on the cytoplasmic matrix. The less virus produced, the more diffuse the antigen found. A striking observation was the presence of viral antigen in dendrites, more precisely on neurotubules and post-synaptic densities. Assembly was observed close to synapses, always on the postsynaptic site, as seen with measles and rhabdoviruses. Infected oligodendrocytes had hypertrophied cytoplasm apposed to some naked axons. Clusters of label were seen in the vacuole area where virus was produced and some particles formed in Golgi vacuoles. With ts 8, viral antigen was rarely observed in neurons of the spinal cord but clusters of small positive cells were seen in the white matter at 3 days PI. Some of these clusters were clearly associated with an area of demyelination. Later (7 days), positive cells were sparse and scattered. EM revealed diffuse cytoplasmic labeling in oligodendrocytes with strong staining in nuclear and cytoplasmic rough ER membranes as well as in several processes extending to the myelinated fibers. So far, evidence of viral antigen in myelin has not been found, but free virions were seen close to myelin. Granulocytes, were found in areas of infection and demyelination and were in contact with myelin debris.

In conclusion, neurons infected with wt can show viral maturation in their dendrites. Proteins might be transported by neurotubules. Propagation of virus from host to presynaptic sites may occur (see in vitro studies). There is a preferential tropism of ts 8 for oligodendrocytes in vivo. Antigen and viruses are present in the oligodendrocyte processes and myelin breakdown occurs early. Some positive cells may persist later but are scattered and more difficult to detect. The main differences between wt and ts 8 induced disease is that neurons are rarely infected with ts 8 so that the animal does not die from involvement of midbrain and other neurons. Oligodendrocyte involvement in both diseases appears identical, at least in the early stage. This involvement leads to progressive demyelination.

Significance of the Program to the Institute: Measles in men, mouse hepatitis virus and VSV in animals, are all involved in acute and chronic infection of the CNS. Basic knowledge of the method in which these viruses modify the cell membrane, first in the simple cell system and secondly in the nerve cell, will help us to understand the pathogenesis of disease.

The mechanisms of viral persistence in the nervous system are still obscure. This year we have analyzed models in which either the nature of the virus (the mutant of mouse hepatitis virus) or the host cell (maturation of neurons in measles infection) influences the outcome of viral infection.

The development of cultures containing myelin-forming cells is a powerful tool to study behavior and structure of these cells first in isolation, and subsequently in association with peripheral or central axons. One can then study the repair

potential of Schwann cells and also examine how virus or various antibodies can alter their function. This is extremely relevant to the study of peripheral demyelination and remyelination in neuropathies in man, and to that of remyelination of central axons by Schwann cells in demyelinating diseases such as multiple sclerosis.

Proposed Course of the Project: Dissociated neuron cultures present many advantages compared to other types of nervous system culture, mainly an accessibility of the cells to homogenous viral infection, to antibody treatment, to immunolabeling and to surface observation with the scanning electron microscope. On the other hand, aggregated brain cultures offer the advantage of more physiological interaction between cells since neuronal transmitter synthesis and myelination are taking place in a large number of cells. Therefore, we would like to extend our studies of interaction between viruses and nerve cells to this system (Dr. Bruce Trapp).

Some of the aspects of viral infection of dissociated neuron cultures (described in Major Findings) need to be studied more in depth. The possibility that interferon might play a role in chronic measles infection of neurons should be explored. In addition, preliminary results seem to indicate that rat pineal cells can be specifically infected with measles virus and that this infection results in a decrease in an enzyme necessary for synthesis of melatonin (in collaboration with Dr. Parfitt). We would like to examine the mechanism by which this enzyme is inhibited in the course of viral infection as a model system. It is indeed possible that, in natural chronic infection of nerve cells, viruses impair function of cells long before killing them.

The differences in maturation of 2 strains of MHV in neuron cultures will be further explored. In order to clarify if the host cell controls these differences, the wt and ts 8 of the JHM strain will be compared to the A59 strain which has a very different passage history (in collaboration with Dr. K. Holmes). We will attempt to characterize the cells in which the virus replicates preferentially, using neuronal and glial markers. We will use monospecific antibody to MHV proteins to determine if the membrane whorls induced by ts 8 contain viral antigens. The details of viral maturation in neurons and non-neuronal cells will be examined with high resolution TEM since preliminary results have demonstrated structural events not identified earlier in other cell types.

We will pursue our study on rat Schwann cells and how they can interact with axons in vivo and in vitro. If their myelinating properties are confirmed, we will study by immunocytochemistry the time sequence of myelin protein synthesis by these cells when they are triggered to myelinate (with Dr. Trapp). Subsequently, we will attempt to study how these events can be altered by viral infection. We will also try to culture Schwann cells of other species such as chicken, mouse, or human, and study various infections of these cells. We will also resume our attempt to grow oligodendrocytes and, possibly, stimulate their mitosis. This would be an extremely useful tool for the study of virus-induced demyelination within the CNS.

Finally, the VIM studies of Dr. Rentier have been extremely productive in revealing the behavior of normal nerve cells in culture. We will now extend these studies to the cytopathic effect of viruses in neuronal and glial cells, using various neurotropic viruses. In addition, the acquisition of an inverted microscope equipped with fluorescence will allow us to follow the fate of labeled viruses and antiviral antibodies interacting with living nerve cells.

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Experimental Therapeutics Branch
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT
October 1, 1979 through September 30, 1980
Experimental Therapeutics Branch, IRP
National Institute of Neurological and Communicative Disorders and Stroke
Donald B. Colne, D.M., F.R.C.P., Chief

Research conducted by the Experimental Therapeutics Branch has continued to focus on the treatment of neurological diseases. As previously, physiology and epidemiology have been pursued in so far as they have been relevant to diseases which are of pharmacological interest. Over the last year, the Behavioral Neuropharmacology Unit was separated administratively from the Experimental Therapeutics Branch, and reestablished as a Neurotoxicology Section. In other respects the resources available to the Experimental Therapeutics Branch have been stable.

Therapeutics Section

1. D-1 and D-2 Receptors

Consideration has been given to the clinical implications of the classification of dopamine receptors into D-1 and D-2 types. Three selective D-2 agonists have now been given to patients with Parkinsonism - bromocriptine, lergotriple and lisuride. All of these agents have been found to possess therapeutic activity. From these findings it is concluded that stimulation of D-2 receptors is of critical importance in the treatment of Parkinson's disease, and it may be inferred that the clinical deficits evident in this disorder arise, at least in part, from inadequate transmission at D-2 receptors.

2. Antiparkinson Efficacy of Lisuride

Twenty Parkinsonian patients have been admitted to hospital for a double-blind study of lisuride. The dose was built up to maximum tolerated levels over 30 days (with an arbitrary upper limit of 5 mg daily), and placebo was substituted for a further 10 days. Subjective and objective (computer assisted) evaluation of clinical deficits revealed improvement in tremor, rigidity and akinesia. Adverse effects were similar to those of other dopaminergic drugs, but somnolence was more prominent. The sedation may be related to the unusual effects of lisuride on serotonergic synapses; this drug appears to be a serotonin agonist in the central nervous system. We are now extending our experience with lisuride in a randomized double-blind study on outpatients, the upper dose limit having been raised to 10 mg daily. Preliminary observations support the general conclusions drawn in the first study. An important practical advantage of lisuride is its relatively simple structure, which should allow production at a substantially lower cost than bromocriptine.

3. Decarboxylation of Levodopa in Man

The rate of decarboxylation of levodopa has been studied in human subjects. Carboxyl labelled (C^{14}) levodopa has been given to Parkinsonian patients and the rate of expiration of labelled carbon dioxide has been measured. The technique, which was pioneered by Carlsson's group in Sweden, is now being applied to the problems of "on-off reactions", the effect of diet on metabolism of levodopa, and the influence of physical exercise on decarboxylation. This work is being conducted in collaboration with Dr. I. Kopin, of NIMH.

4. Objective Methods of Quantifying Movement Disorders

Computer assisted methods have been employed to study the speed and precision of hand movement, the velocity of gait and mean step length. These techniques are sufficiently simple and versatile to be employed for the clinical evaluation of drugs, and have proved most helpful in assessing the response of Parkinsonian patients to lisuride. This work has been performed in collaboration with Dr. E. Evarts, NIMH.

5. Studies of Eye Movements with and without Visual Feedback

The speed and accuracy of eye movements have been investigated with and without visual feedback of information. This study was undertaken to obtain evidence bearing on the claim that the fundamental physiological defect in Parkinsonism is impairment of the programming of movement. Parkinsonians and control subjects responded in a similar way to loss of visual feedback, implying that the programming process is normal in Parkinson's disease.

6. "Simple and "Choice" Reaction Times in Parkinson's Disease

In order to investigate further the hypothesis of defective motor programming in Parkinsonism, we have compared the reaction times in patients who have (a) a simple task, e.g., "turn your hand in a clockwise direction", or (b) a more complex task involving a choice, e.g., "turn your hand in the direction indicated by a light illuminating on your right or your left". There was no significant difference between the patients and controls, from which it may be inferred that Parkinsonians do not have a specific problem in programming movements.

7. Influence of Posture on Movement in Parkinsonism

It has been suggested that akinesia and bradykinesia might stem from difficulty in coordinating postural control and voluntary movement. To investigate this hypothesis, the initiation of fast voluntary eye movement was studied during rotation of the body and head. There was no significant difference between Parkinsonian patients and controls, indicating that there is no major difficulty in superimposing volitional motor activity over postural motor mechanisms.

8. Neuroendocrinological Observations

There has been considerable controversy over the location of the pathology of dopaminergic neurons in Parkinson's disease; is it confined to the nigrostriatal pathway? We have attempted to answer this question by studying the effects of dopaminergic agents on the control of mammatroph cells in the pituitary of 13 untreated Parkinsonian patients and 12 age matched controls. We have stimulated prolactin release by injection of thyrotropin releasing hormone, and we have measured the attenuation of this response by bromocriptine and levodopa (with and without carbidopa). The inhibitory effect of dopaminergic agents was increased in Parkinson's disease, suggesting that there is, indeed, a chronic reduction of dopaminergic influences on the pituitary, with compensatory up-regulation. This work was performed in collaboration with Dr. M. Thorner, of the University of Virginia Medical School.

9. Blood-brain Barrier in the Substantia Nigra of Gerbils

It has been proposed that the nigrostriatal pathway may be selectively vulnerable because of relatively high capillary permeability to circulating toxins, which might in turn derive from a localized increase in the sensitivity of nigral capillaries to non-specific damage. We have injected the cell poison chloromercuribenzoic acid into the aorta of mongolian gerbils. Prominent extravasulation of microperoxidase was subsequently detected in the cerebral cortex and striatum, but not in the substantia nigra. This work was performed in collaboration with Dr. H. Hervonen of the University of Tampere, Finland.

10. Concordance of Parkinsonism in Twins

We have continued to ascertain and evaluate pairs of twins where Parkinsonism has been diagnosed. We now have information on 30 pairs, and the concordance rate remains surprisingly low - only one definite pair. This result supports the view that the search for an etiology should be focused on environmental functions to which patients are exposed after childhood. This work has been conducted in collaboration with Dr. R. Eldridge, NINCDS, and Dr. R. Duvoisin, Rutgers Medical School.

Neuropharmacology Section

1. Stable Isotope Studies of Transmitter Metabolism

Previous investigations have shown that inhalation of a breathing mixture containing a non-radioactive isotope of oxygen ($^{18}\text{O}_2$) produces readily detectable labelling of major monoamine metabolites in plasma, urine, and cerebrospinal fluid. Sufficient quantities of oxygen-18 became available during the past year to study several additional patients. The results provided further evidence to suggest that the central nervous system of parkinsonian patients contains a relatively small pool of dopamine which turns over at a rapid rate. The possibility that a compensatory hyperactivity of surviving dopamine neurons occurs in parkinsonian patients could have important implications for the rational development of improved drug therapy for this disorder. Future studies will examine the effects of dopamine agonists and antagonists on central dopamine metabolism, and explore central monoamine metabolism in other neurological disorders as well as in individuals who are free of nervous system disease.

2. Cerebrospinal Fluid Studies

An improved agarose gel electrophoretic technique was used to fractionate serum and CSF proteins in patients with senile dementia of the Alzheimer type. The CSF electrophoretic patterns demonstrated three abnormal bands in the gamma-globulin region in a majority of individuals studied. Since these bands did not occur in patients with Parkinson's disease or Huntington's chorea, it is unlikely that they simply reflect neuronal degeneration. Although the antigenic specificities of the bands are unknown, possibilities include the etiologic agent itself or a component of brain tissue. The presence of these bands supports hypotheses involving an infectious agent or abnormal regulation of the immune system in the etiology of Alzheimer's disease.

3. Positron Emission Tomography Studies

Investigations of regional neuronal activity in patients with various dementing disorders are currently being conducted by means of positron emission tomography following ^{18}F -2-fluoro-2-deoxyglucose administration. The accumulation of the labelled glucose analogue is used to provide an index of local neuronal function in patients with Alzheimer's disease or Huntington's chorea. Clinically normal individuals, at risk for Huntington's disease, serve as controls. Attempts are being made to correlate aspects of cognitive function under basal conditions as well as during activation by various mental tasks or by drugs which influence higher cortical function. These studies will be extended to patients with hyperkinetic extrapyramidal disorders, such as tardive dyskinesia, Tourette syndrome, and dystonia musculorum deformans, where no neuro-pathological alterations have been found to account for neurologic symptoms.

4. Dopamine Agonist Therapy of Neuropsychiatric Disease

Efforts have continued during the past year to select, procure, and evaluate dopamine agonists which may be beneficial in disorders thought to reflect dopaminergic hyperfunction. The current working hypothesis - supported by earlier observations with apomorphine, bromocriptine, CF 25-397, and piribedil - is that dopamine agonists, which preferentially stimulate certain presynaptic dopamine receptors, inhibit dopaminergic transmission and thus ameliorate symptoms of hyperdopaminergic states. Such a mechanism may be peculiarly dose dependent, prompting in recent months extensive dose-response tests of piribedil. Other clinical studies currently being implemented in patients with tardive dyskinesia and related hyperkinetic extrapyramidal disorders include a trial of apomorphine in combination with a peripheral dopamine receptor blocking agent to limit gastrointestinal side effects, as well as tests of N-propyl-norapomorphine and S-3608, non ergot dopaminergic agonists selected on the basis of their biochemical and pharmacologic profiles in the experimental animal.

5. GABAmimetic Therapy of Neuropsychiatric Disease

Biochemical and pharmacologic observations link GABA system alterations with several central nervous system disorders, and suggest that augmentation of GABA mediated synaptic function might suppress symptoms of naturally occurring or drug induced hyperkinetic extrapyramidal disorders. Recent clinical studies by this Section with the potent GABA agonist, muscimol, support this view. Since toxic effects of the drug limit clinical usefulness, clinical trials of two novel GABA mimetic compounds are now beginning in patients with tardive dyskinesia: SL76002, which acts indirectly by increasing the availability of GABA at central synapses or by directly stimulating post-synaptic GABA receptors; and gamma vinyl GABA, which elevates brain GABA by blocking its degradatory enzyme, GABA transaminase. Future plans also call for clinical trials of muscimol in combination with a short acting benzodiazepine derivative, based upon preclinical observations indicating that certain behavioral effects of GABA agonists are potentiated by benzodiazepines.

6. Cholinergic Agonists Therapy of Neuropsychiatric Disease

A reduction in cortical choline acetyltransferase activity now constitutes the most consistent evidence of a transmitter abnormality in patients with senile dementia of the Alzheimer type. Attempts to treat this disorder with acetylcholine precursors have yielded disappointing results, possibly due to the loss of choline acetyltransferase

containing neurons required to convert the exogenous precursor to the active transmitter. Since muscarinic receptors appear relatively well preserved in Alzheimer type patients, direct acting cholinergic agonists might prove more effective. Studies carried out with one muscarinic agonist, arecoline, have as yet failed to provide unequivocally beneficial results. One difficulty in demonstrating efficacy derives from the inadequacy of available psychometric instruments, especially in situations requiring their serial administration. An improved test battery has now been devised, and studies of arecoline in Alzheimer's disease will be completed during the coming year.

7. Peptide Studies

The limited efficacy and potential toxicity of neuroleptics have prompted the search for alternative pharmacologic approaches to the treatment of hyperkinetic extrapyramidal disorders. Recent evidence suggests that fragments of the pituitary hormone β -lipotropin (β -LPH) exert behavioral effects not mediated by opiate receptors. One such fragment, des-tyrosine- γ -endorphin (β -LPH₆₂₋₇₇; DT γ E), exhibits neuroleptic-like activity in the experimental animal but, in contrast to all existing neuroleptics, without directly interfering with dopaminergic transmission. Preliminary and largely uncontrolled clinical observations with DT γ E in Europe suggest the peptide may have antipsychotic activity. In order to evaluate both antidyskinetic and antipsychotic potency, DT γ E has been administered to otherwise untreated chronic schizophrenic patients with and without associated tardive dyskinesia. Preliminary results from this double blind, placebo controlled trial suggest no consistent alteration in behavioral, motor, or endocrinologic function. Extension of this work to shorter sequence β -endorphin derivatives (for example, β -LPH₆₆₋₇₇), also reported to have neuroleptic-like activity, is anticipated.

The therapeutic implications of recently emerging preclinical information regarding the influence of the vasopressin system on learning and memory also received investigative attention during the past year. In the experimental animal, the systemic administration of vasopressin facilitates complex cognitive tasks including the acquisition and maintenance of learned responses. The discovery that vasopressin nerve cell bodies in the supraoptic nucleus project to the hippocampus and other cortical areas, may provide some anatomic basis for these observations. Treatment with lysine vasopressin has been reported in small, uncontrolled European studies to improve aspects of attention and memory in individuals with such disorders as post-traumatic amnesia and senile dementia. Preliminary results of the Section's current double blind, placebo controlled study of lysine vasopressin in Alzheimer disease patients suggest that some cognitive processes may be improved. Depending on the final outcome, this work may be extended to include a promising new vasopressin analogue, desglycinamide-arginine vasopressin (DGAVP), which retains the ability to influence learned responses in the laboratory animal but is relatively free of antidiuretic or pressor activity.

Clinical Epilepsy Section

I. Diagnostic and Therapeutic Reevaluation of Patients with Intractable Epilepsy

The Clinical Epilepsy Section is interested in improvement of seizure control, reduction of drug-induced side effects, and improved potential for rehabilitation by the utilization of newly developed intensive monitoring techniques. These include simultaneous video recording of seizures, long-term telemetering of EEGs, and daily determinations of antiepileptic drug concentrations.

A long neglected area that has been investigated by intensive monitoring is the psychogenic (hysterical) seizure, a major diagnostic problem. Intensive monitoring of patients with intractable seizures has uncovered a previously unsuspected frequency of psychogenic attacks.

Another important aspect in the treatment of patients with intractable epilepsy is the permanence of the benefit of intensive monitoring. At reevaluation after two years, 65% of the 46 patients with severe epilepsy still showed improved seizure control compared with preadmission frequency, 63% had reduced drug toxicity, and 41% had improved social adjustment. It is important to establish which patients might be best helped with the greatest long-term improvement in their seizure problems.

An important development in the evaluation of patients with intractable seizures is the new technique of positron emission tomography. Studies are now underway in patients under a variety of therapies with intractable multifocal seizures, partial seizures, and primary generalized seizures.

The recent utilization of evoked responses in patients with epilepsy has opened a new field for studies of patients with intractable seizures. Preliminary data suggest that the dominant eye may greatly influence the amplitude of the visual evoked response. These subtle changes may be wrongly interpreted in patients with partial seizures unless this asymmetry is accounted for. In addition, patients with complex partial seizures are currently being evaluated for abnormalities of the visual evoked response, auditory and brainstem evoked potentials, and the somatosensory evoked potentials.

The video-taped seizures at the Clinical Epilepsy Section have formed the basis of an unparalleled library of seizures for teaching and analysis. The video-taped seizures formed the integral part of an education film, "Differential Diagnosis of Complex Partial Seizures", now being distributed. The Clinical Epilepsy Section is constantly making technical advances in intensive monitoring, both at the electronic and the pharmacologic level.

2. Clinical Pharmacology of Antiepileptic Drugs

Pharmacologic projects are underway and are described in the following paragraphs.

The effect of food on the absorption of antiepileptic drugs is a recently developed project. Although interest has recently developed on the effect of food on absorption of a wide variety of medications, little evidence has thus far accumulated on the effect of food on antiepileptic drugs. Initial studies performed on volunteers have suggested that food may affect the absorption of carbamazepine. In the studies conducted by the Clinical Epilepsy Section the effect of taking medication with food and without food has been analyzed in seven patients. Steady state levels obtained over a two-week therapy, as well as after single administration, have been analyzed. Although steady state levels do not appear to change, food does appear to affect the rate of absorption of carbamazepine. These preliminary findings suggest that food may be an important factor in the absorption of medications in individual patients, and that careful drug administration may greatly influence the total dose of drug that a patients can tolerate.

Recognition of their subtle but significant toxicity has made the need for sedative-hypnotic antiepileptic drugs less certain, but their removal is often thought to be difficult and dangerous. Barbiturates and benzodiazepines were completely withdrawn from 66

patients with intractable epilepsy (39 inpatients and 27 outpatients). After 6 months of outpatient follow-up, 59 patients were still on a nonsedative regimen: 47 showed improvement in either toxicity or seizure control or both. This study shows that sedative drugs are not necessary for optimal seizure control, even in intractable epilepsy, and that they may be safely withdrawn.

Valproic acid is a new drug which is structurally unrelated to conventional anticonvulsant drugs. However, when valproic acid is administered in conjunction with phenobarbital, it potentiates side effects of the latter. We investigated the mechanisms for the alteration of valproate in the serum level of phenobarbital by mass spectroscopy. Early data suggested that the primary effect was on altered metabolism of phenobarbital rather than by increased renal absorption or changes in the volume of distribution. More recently, we have studied the interaction of valproic acid with acetaminophen. Both agents are partially metabolized by conjugation with glucuronide. In these studies, acetaminophen metabolism does not appear to be affected by chronic therapy with valproic acid, thereby demonstrating that the conjugation for these two agents is different, and strongly suggesting that the inhibition of phenobarbital metabolism by valproic acid is at the hydroxylation reaction.

Biochemical Pharmacology Unit

I. Biochemical Studies of Dopaminergic Receptors

When the existence of two distinct categories of dopamine receptors was initially proposed, the mammothrophs of the anterior pituitary gland provided the best example of a cell possessing a D-2 receptor. However, conclusions about the physiological responses of the mammothrophs or the biochemical basis of these responses were deduced from indirect measurements. Thus, the anterior pituitary gland is a heterogeneous tissue, composed of several distinct types of cells; depending upon hormonal factors, the mammothrophs represent between 5% and 50% of the total cells in the anterior hypophysis. Because prolactin is a mammothroph-specific hormone, the presence of a dopamine receptor upon mammothrophs and the independence of the receptor from a cyclic AMP generating system was deduced by determining the ability of drugs to alter prolactin release from intact or dispersed pituitary glands. Direct determination of any biochemical parameter of the mammothrophs, which was also common to the other cell types in the anterior pituitary, was complicated because the "signal" from the mammothrophs was lost in the "noise" generated by the other cells in the gland.

A new experimental system was required to study directly the biochemistry and physiology of the D-2 receptor. Among the requirements of the system were : 1) the presence of a relatively homogeneous tissue; 2) the presence of a presumptive D-2 receptor and a well-defined response to dopamine; and 3) the presence of a well-defined physiological role for cyclic AMP.

The intermediate lobe of rat pituitary gland (IL) provides an easily accessible tissue for studying a D-2 dopamine receptor. The intermediate lobe is a relatively homogeneous tissue, by histological or cytochemical criteria, containing alpha-melanocyte stimulating hormone (alpha-MSH). The physiological response of the intermediate lobe to dopamine, an inhibition of the release of alpha-MSH, can be easily quantified. A beta-adrenoceptor also occurs on the parenchymal cells of the intermediate lobe. The properties of the beta-adrenoceptor, the enhancement of adenylate cyclase activity and the enhanced release of alpha-MSH as a consequence of beta-adrenergic stimulation have been

characterized in detail in the past two years. The results are compatible with the possibility that occupancy by agonists (i.e. stimulation) of the beta-adrenoceptor enhances adenylate cyclase activity, resulting in an accumulation of cyclic AMP which initiates the intracellular events that are ultimately expressed as an enhanced release of alpha-MSH.

The dopamine receptor in the intermediate lobe has also been extensively studied in the past year. Stimulation of the dopamine receptor diminishes basal release of alpha-MSH as well as the L-isoproterenol-induced accumulation of cyclic AMP, and the L-isoproterenol-enhanced release of alpha-MSH. The pharmacology of the dopamine receptor can be characterized on the basis of the ability of drugs to mimic or block any of these effects of dopamine; however, the dopamine-induced decrease in the responsiveness of the beta-adrenoceptor to L-isoproterenol has provided the basis for our characterization of the pharmacology of the dopamine receptor. The dopamine receptor recognizes dopamine, apomorphine and the dopaminergic ergots as agonists, and is antagonized by a variety of dopamine antagonists, including (-) sulpiride, a substituted benzamide which selectively blocks D-2 receptors; therefore, the dopamine receptor in the IL has been assigned to the D-2 category. The biochemical basis of the decreased responsiveness of the beta-adrenoceptor was investigated; apomorphine was the dopaminergic agonist of choice for these studies because it does not interact with the beta-adrenoceptor. Stimulation of the D-2 receptor diminishes the ability of GTP to enhance either basal or L-isoproterenol-stimulated adenylate cyclase activity. The decreased efficacy of GTP is reflected as a non-competitive inhibition of the responsiveness of the beta-adrenoceptor.

In summary, the concept of the existence of a D-2 receptor has been extended in the past two years. Two years ago the D-2 receptor was a theoretical construct, occurring on cells which were not amenable to direct experimental examination. Today, the concept of the D-2 receptor is backed up by direct experimental observations. Furthermore, information is now available as to how the D-2 receptor works (at least in decreasing the responsiveness of the beta-adrenoceptor in the intermediate lobe of the rat pituitary gland). This information provides a starting point for experimental observations in the brain and other tissues where D-2 receptors also occur. However, because the intermediate lobe is innervated by dopaminergic neurons whose soma lie in the arcuate nucleus, dopaminergic neurotransmission can be studied to advantage in the intermediate lobe. In this regard, the intermediate lobe may be as useful for the investigation of dopaminergic neurotransmission as has been the case of sympathetic ganglia or the myoneural junction and cholinergic neurotransmission.

Physiological Neuropharmacology Unit

I. Dopamine Autoreceptors and the Striatonigral "Feedback Loop" in Regulation of Dopamine Activity: Effects of Dopamine Agonists

Neurological investigation of the effects of systemically administered dopamine agonists continue to suggest that the ergot agonists have effects on the central dopamine system which distinguish them from classic dopamine agonists such as apomorphine. Previously, we found that systemically administered apomorphine, a dopamine agonist which normally depresses the firing of dopamine neurons, still inhibits the activity of the dopamine cells after a kainic acid induced lesion of the striatonigral pathway. Lisuride, an ergot derivative which also inhibits dopamine cell activity when administered systemically to normal rats, was less effective in lowering dopamine cell firing rates in animals with

unilateral kainic-acid induced lesions of the striatonigral pathway. We concluded from these observations that lisuride was less effective than apomorphine at stimulating dopamine receptors on the dopamine cell body and was probably acting, at least in part, through dopamine receptors in the striatum and the striatonigral feedback loop to inhibit dopamine cell activity. We have, however, recently made the surprising observation that the effects of lisuride on dopamine firing rates are also attenuated on the side opposite the lesion in rats which have received unilateral kainic acid injections in the striatum. Since there is no obvious cell loss on the side of the brain contralateral to the kainic acid injection, and no known crossover of the brain's two striatonigral pathways, this finding has interesting implications for the mechanism of action of this ergot and factors regulating the activity of the dopamine neurons. Some part of the brain affected by the unilateral kainic acid injections, or some other consequence of the injection, appears able to modify the effects of lisuride on dopamine cell activity on the intact side of the brain.

2. Role of Peptides in the Basal Ganglia: Cholecystokinin and Dopamine Neurons

Preliminary observations have suggested that systemically administered cholecystokinin (CCK), a peptide which appears to be synthesized in some dopamine neurons, causes a decrease in the ability of amphetamine to inhibit dopamine activity. This has suggested that the peptide may attenuate dopamine release. We have also found that reserpine treatment decreases CCK levels in the accumbens, but not the striatum, an observation consistent with the distribuion of CCK-containing dopamine cells.

3. The Role of GABA and Effects of GABAmimetics in the Substantia Nigra and A10 Dopaminergic Region

We have investigated the effects of two GABAmimetics, muscimol and 4,5,6,7-tetrahydro isoxazola-[5,4-c]-pyridin-3-ol (THIP), on the activity of the A10 dopamine cells in the ventral tegmental area. The effects on the A10 dopamine neurons of systemic administration of these drugs are very similar to those observed with the A9 dopamine neurons in the pars compacta of the substantia nigra. They both caused dose-related increases in extracellular single unit activity; muscimol was approximately 15-20 times more potent than THIP. These results disprove the hypothesis that GABA agonists should inhibit the A10 dopamine neurons, a prediction based on evidence that GABAergic neurons exert a direct inhibitory influence on the A10 cells. The results suggest that the systemically administered GABA agonists influence other neurons or pathways whose effects upon dopamine cell firing are more predominant than the direct effects of these agonists on the dopamine cell body. They contribute to the notion that the nigrostriatal (A9) and mesolimbic (A10) dopamine systems may be functionally as well as anatomically parallel and analogous.

We have continued to examine the development of GABAergic supersensitivity in the substantia nigra pars reticulata after kainic acid-induced lesion of the striatonigral GABAergic pathway.

Iontophoresis of GABA and muscimol onto the reticulata cells in the lesioned rats showed that these cells were significantly more sensitive to GABA than were the controls. Since reticulate cells in kainic acid-lesioned rats do not demonstrate supersensitivity to i.v. muscimol, their increased response to iontophoresed GABA and the reported increases in GABA binding may not be predictive of physiologically important or clinically significant changes in reticulata cell function in diseases where striatonigral GABA pathways degenerate.

4. Effects of Neurotransmitter Agonists and Antagonists Upon the Activity of Globus Pallidus Neurons

Dopamine agonists and antagonists have been shown to have marked effects on the firing rates of the nigrostriatal dopamine neurons. Changes in the activity of striatal neurons have also been observed. In the past year we have been examining the effects of systemic administration of apomorphine, amphetamine and haloperidol on the single unit activity of the cells in the globus pallidus, an area receiving a major input from the striatum. Haloperidol alone caused a slight but significant decrease in the activity of pallidal cells in locally anesthetized, paralyzed and artificially respired rats. Apomorphine increased the activity of 1/3 of the pallidal cells at doses greater than those needed to alter the activity of nigrostriatal dopamine neurons. Amphetamine caused a much more dramatic and consistent increase in pallidal activity which was attenuated by reserpine and α -methyl-para tyrosine pretreatment. The increases in activity caused by apomorphine and amphetamine were reversed by haloperidol and, in the apomorphine, but not in the amphetamine, pretreated rats, haloperidol reduced firing to near or total inhibition. It appears that apomorphine may be causing a subtle change in dopaminergic modulation of globus pallidus transmission that is enhancing the ability of haloperidol to inhibit firing. In addition, we have observed that carbachol produces an increase in 3/4 of the cells recorded and muscimol consistently inhibits pallidal activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 NS 02258-04 ET
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PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less) Therapeutic Studies in Parkinsonism and Other Movement Disorders
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NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT				
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COOPERATING UNITS (if any)	Laboratory of Clinical Science, NIMH; Laboratory of Neurophysiology, NIMH; Biochemical Pharmacology Section, HE, NHLBI; Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia; Department of Neurology, Rutgers University School of Medicine; University of Tampere, Finland ; NES, NINCDS
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LAB/BRANCH Experimental Therapeutics Branch
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SECTION Therapeutics Section

INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4.5	PROFESSIONAL: 4.5	OTHER: 0
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		

SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this study is to investigate the possible efficacy and safety of new compounds for the treatment of certain disorders of movement, and to employ drugs as tools to analyze the physiological and pharmacological mechanisms mediating various motor deficits. The main conclusions deriving from observations over the last year are: (1) The neurological deficits of Parkinsonism stem, at least in part, from inadequate transmission at D-2 receptors, which can be alleviated by selective D-2 agonists. (2) Lisuride has antiparkinson efficacy, with adverse actions similar to bromocriptine but more prominent induction of somnolence. (3) Administration of carboxyl labelled levodopa is a convenient approach to monitoring dopamine formation in Parkinsonian patients. (4) Dopaminergic systems outside the nigrostriatal pathway are abnormal in Parkinson's disease; there is increased sensitivity of D-2 receptors on lactotroph cells in the pituitary. (5) The programming of voluntary movement appears to be normal in Parkinson's disease. (6) The relationship between voluntary and postural motor mechanisms is normal in Parkinson's disease. (7) The capillaries forming the blood-brain barrier of the substantia nigra are less susceptible to chemical damage than those of the cerebral cortex and striatum.

Project Description:Objectives:

This project is designed to improve treatment and elucidate the pharmacological, physiological and biochemical abnormalities occurring at synaptic level in certain neurological diseases.

Methods:

Inpatients and outpatients are studied. Specimens of body fluids (including CSF) are taken for assay of transmitters, their metabolites, drugs, and routine biochemical and haematological indices of pharmacotoxicity. Motor control is studied by measuring velocity and force of movement, integrated electromyographic activity, and by conventional clinical scoring techniques that involve careful history taking and physical examination. Where possible the patient is used as his own control by making observations during different (blind) therapeutic regimens, and by studying asymmetric motor deficits.

Major Findings and Significance to Biomedical Research and the Program of the Institute:1) D-1 and D-2 Receptors

Consideration has been given to the clinical implications of the classification of dopamine receptors into D-1 and D-2 types. Three selective D-2 agonists have now been given to patients with Parkinsonism - bromocriptine, lergotril and lisuride. All of these agents have been found to possess therapeutic activity. From these findings it is concluded that stimulation of D-2 receptors is of critical importance in the treatment of Parkinson's disease, and it may be inferred that the clinical deficits evident in this disorder arise, at least in part, from inadequate transmission at D-2 receptors.

2) Antiparkinson Efficacy of Lisuride

Twenty Parkinsonian patients have been admitted to hospital for a double-blind study of lisuride. The dose was built up to maximum tolerated levels over 30 days (with an arbitrary upper limit of 5 mg daily), and placebo was substituted for a further 10 days. Subjective and objective (computer assisted) evaluation of clinical deficits revealed improvement in tremor, rigidity and akinesia. Adverse effects were similar to those of other dopaminergic drugs, but somnolence was more prominent. The sedation may be related to the unusual effects of lisuride on serotonergic synapses; this drug appears to be a serotonin agonist in the central nervous system. We are now extending our experience with lisuride in a randomized double-blind study on outpatients, the upper dose limit having been raised to 10 mg daily. Preliminary observations support the general conclusions drawn in the first study. An important practical advantage of lisuride is its relatively simple structure, which should allow production at a substantially lower cost than bromocriptine.

3) Decarboxylation of Levodopa in Man

The rate of decarboxylation of levodopa has been studied in human subjects. Carboxyl labelled (C14) levodopa has been given to Parkinsonian patients and the rate of expiration of labelled carbon dioxide has been measured. The technique, which was pioneered by Carlsson's group in Sweden, is now being applied to the problems of "on-off reactions", the effect of diet on metabolism of levodopa, and the influence of physical exercise on decarboxylation. This work is being conducted in collaboration with Dr. I. Kopin, of NIMH.

4) Objective Methods of Quantifying Movement Disorders

Computer assisted methods have been employed to study the speed and precision of hand movement, the velocity of gait and mean step length. These techniques are sufficiently simple and versatile to be employed for the clinical evaluation of drugs, and have proved most helpful in assessing the response of Parkinsonian patients to lisuride. This work has been performed in collaboration with Dr. E. Evarts, NIMH.

5) Studies of Eye Movements with and without Visual Feedback

The speed and accuracy of eye movements have been investigated with and without visual feedback of information. This study was undertaken to obtain evidence bearing on the claim that the fundamental physiological defect in Parkinsonism is impairment of the programming of movement. Parkinsonians and control subjects responded in a similar way to loss of visual feedback, implying that the programming process is normal in Parkinson's disease.

6) "Simple and "Choice" Reaction Times in Parkinson's Disease

In order to investigate further the hypothesis of defective motor programming in Parkinsonism, we have compared the reaction times in patients who have (a) a simple task, e.g., "turn your hand in a clockwise direction", or (b) a more complex task involving a choice, e.g., "turn your hand in the direction indicated by a light illuminating on your right or your left". There was no significant difference between the patients and controls, from which it may be inferred that Parkinsonians do not have a specific problem in programming movements.

7) Influence of Posture on Movement in Parkinsonism

It has been suggested that akinesia and bradykinesia might stem from difficulty in coordinating postural control and voluntary movement. To investigate this hypothesis, the initiation of fast voluntary eye movement was studied during rotation of the body and head. There was no significant difference between Parkinsonian patients and controls, indicating that there is no major difficulty in superimposing volitional motor activity over postural motor mechanisms.

8) Neuroendocrinological Observations

There has been considerable controversy over the location of the pathology of dopaminergic neurons in Parkinson's disease; is it confined to the nigrostriatal pathway? We have attempted to answer this question by studying the effects of dopaminergic agents on the control of mammotroph cells in the pituitary of 13 untreated Parkinsonian patients and 12 age matched controls. We have stimulated prolactin release by injection of thyrotropin releasing hormone, and we have measured the attenuation of this response by bromocriptine and levodopa (with and without carbidopa). The inhibitory effect of dopaminergic agents was increased in Parkinson's disease, suggesting that there is, indeed, a chronic reduction of dopaminergic influences on the pituitary, with compensatory up-regulation. This work was performed in collaboration with Dr. M. Thorner, of the University of Virginia Medical School.

9) Blood-brain Barrier in the Substantia Nigra of Gerbils

It has been proposed that the nigrostriatal pathway may be selectively vulnerable because of relatively high capillary permeability to circulating toxins, which might in turn derive from a localized increase in the sensitivity of nigral capillaries to non-specific damage. We have injected the cell poison chloromercuribenzoic acid into the aorta of mongolian gerbils. Prominent extravasulation of microperoxidase was subsequently detected in the cerebral cortex and striatum, but not in the substantia nigra. This work was performed in collaboration with Dr. H. Hervonen of the University of Tampere, Finland.

10) Concordance of Parkinsonism in Twins

We have continued to ascertain and evaluate pairs of twins where Parkinsonism has been diagnosed. We now have information on 30 pairs, and the concordance rate remains surprisingly low - only one definite pair. This result supports the view that the search for an etiology should be focused on environmental functions to which patients are exposed after childhood. This work has been conducted in collaboration with Dr. R. Eldridge, NINCDS, and Dr. R. Duvoisin, Rutgers Medical School.

Proposed Course:

- 1) The long term efficacy and toxicity of lisuride will be studied in Parkinson's disease.
- 2) A new D-2 agonist, CU 32085, will be investigated as a potential antiparkinson agent.
- 3) Decarboxylation of levodopa will be monitored in Parkinsonian patients during "on-off" reactions, high protein meals, and physical exertion.
- 4) The actions of exogenously administered hydroxylase cofactor (tetrahydrobiopterin) will be studied in man.

5) The anatomy of the substantia nigro will be compared in black and white subjects without Parkinson's disease, in an attempt to elucidate the powerful racial difference in the prevalence of the disorder.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02265-04 ET
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Pharmacology, Biochemistry and Physiology of Central Neurotransmitters		
NAMES, LABRATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	Thomas N. Chase	Chief, Pharmacology Section ETB MINCDS
OTHER:	Raymon Durso Carol A. Tamminga	Clinical Associate ETB MINCDS Guest Worker ETB MINCDS
COOPERATING UNITS (if any) B.L. Beasley, U.S.P.H.S. Hospital, Staten Island; G. Sedvall, Karolinska Institute, Stockholm; D. Samuel, Weizmann Institute, Rehovot.		
LAB/BRANCH Experimental Therapeutics Branch		
SECTION Pharmacology Section		
INSTITUTE AND LOCATION MINCDS, NIH, Bethesda, Maryland 20205		
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<input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this project is to develop improved <u>drug therapies for nervous system disease</u> . Towards this end, investigations seek to determine the relationship between <u>dysfunction in a specific neurotransmitter system</u> and the <u>appearance of a particular neurologic syndrome</u> . In addition, the ability of novel pharmacologic agents to modify the activity of specific transmitter systems in brain and spinal cord are investigated. Major topics under current study include:		
1) Application of the oxygen-18 method for evaluating central monoamine metabolism and the fluorodeoxyglucose method combined with positron emission tomography to assess regional transmitter function in man. 2) Relation of monoamine- and peptide- containing neural system activity to specific motor, sensory, behavioral, or endocrinologic functions. 3) Ability of selective agonists and antagonists of receptors in systems mediated by dopamine, serotonin, acetylcholine, or gamma aminobutyric acid to influence neurologic function.		

Objectives and Methods Employed:

The Section directs its principal research efforts towards the rational development of improved drug treatments for nervous system disease. On the basis of an integrated program of clinical and preclinical studies, attempts are made to relate the activity of a particular transmitter system to a specific centrally mediated function. Novel pharmacologic agents are then evaluated in animal models and in man for their ability to selectively modify these systems and thus ameliorate certain neurologic or psychiatric symptoms. The principal focus is on dopaminergic mechanisms in relation to hyperkinetic extrapyramidal disorders; the role of the dopamine system, as well as other transmitter systems with which it interacts, in the regulation of motor, behavioral, and endocrinologic functions are also investigated.

Major Findings and Proposed Course:

1. Stable isotope studies of transmitter metabolism

Previous investigations have shown that inhalation of a breathing mixture containing 79% nitrogen and 21% oxygen (95% $^{18}\text{O}_2$) can produce readily detectable labeling of major monoamine metabolites in plasma, urine and cerebrospinal fluid. Sufficient quantities of oxygen-18 became available during the past year to study several additional patients. The results provided further evidence to suggest that the central nervous system of parkinsonian patients contains a relatively small pool of dopamine which turns over at a rapid rate. The possibility that a compensatory hyperactivity of surviving dopamine neurons occurs in parkinsonian patients could have important implications for the rational development of improved drug therapies for this disorder. Future studies will examine the effects of dopamine agonists and antagonists on central dopamine metabolism, and examine central monoamine metabolism in certain other neurological disorders (especially tardive dyskinesia) as well as in individuals who are free of nervous system disease.

2. Cerebrospinal Fluid Studies

An improved agarose gel electrophoretic technique has been used to fractionate serum and CSF proteins in patients with presenile dementia of the Alzheimer type. The CSF electrophoretic patterns demonstrated three abnormal bands in the gamma-globulin region in a majority of patients studied. Since these bands were not seen in patients with idiopathic Parkinson's disease or Huntington's chorea, it is unlikely that they merely reflect neuronal degeneration. They probably represent oligoclonal production of IgG plasmacytes, and are evidence of humoral immunologic responses despite normal absolute immunoglobulin levels. Because these bands were not present in serum, production was probably taking place within the central nervous system. Although the antigenic specificities of the bands are unknown, possibilities include the etiologic agent itself or a component of brain tissue. The presence of these bands supports hypotheses involving an infectious agent or abnormal regulation of the immune system in the etiology of Alzheimer's disease. This in-

vestigation will not be extended, and future CSF studies will largely be directed towards the evaluation of central peptidergic function in relation to various neuropsychiatric disorders.

3. Dopamine agonist therapy of neuropsychiatric disease

Efforts have continued during the past year to select, procure, and evaluate certain dopamine agonists which may be beneficial in disorders attributed to dopamine subsystem hyperfunction. Our working hypothesis, supported by earlier observations with apomorphine, bromocriptine, CF25-397, and piribedil, is that dopamine agonists which preferentially stimulate certain presynaptic dopamine receptors, may inhibit dopaminergic transmission and thus ameliorate symptoms of hyperdopaminergic disorders. Such a mechanism may be peculiarly dose dependent, and in recent months careful dose-response tests of piribedil have been undertaken to more fully evaluate this possibility. Other clinical studies currently being implemented in patients with tardive dyskinesia and related hyperkinetic extrapyramidal disorders include a trial of apomorphine in combination with a peripheral dopamine receptor blocking agent to limit gastrointestinal side effects; tests of n-propylnorapomorphine and S-3608, both non-ergot dopaminergic agonists selected on the basis of their biochemical and pharmacologic profiles in the experimental animal. In addition, several other putative dopamine agonists (including RU24213, a long acting congener of n-phenylethyl-m-tyramine) are now being evaluated preclinically.

4. GABAmimetic therapy of neuropsychiatric disease

Biochemical and pharmacologic observations tend to link GABA system alterations with various central nervous system disorders, and suggest that augmentation of GABA mediated synaptic function might suppress symptoms in patients with tardive dyskinesia and related naturally occurring or drug induced hyperkinetic extrapyramidal disorders. Recent clinical observations by this Section with the potent GABA agonist, muscimol, support this view. Since muscimol toxicity limits clinical usefulness, we are now beginning further neuropharmacologic studies of the GABA system using two novel GABAmimetic compounds: SL76002, which stimulates GABA transmission by either increasing the availability of GABA at central synapses or by directly stimulating postsynaptic GABA receptors, and γ -vinyl GABA, which elevates brain GABA levels by blocking its degradatory enzyme, GABA transaminase. Future plans also call for clinical trials of muscimol in combination with a short acting benzodiazepine derivative, based upon recent preclinical observations indicating that certain of the behavioral effects of GABA agonists can be substantially potentiated by benzodiazepines.

5. Cholinergic agonist therapy of neuropsychiatric disease.

A reduction in cortical choline acetyltransferase activity now constitutes the most consistent evidence of a transmitter abnormality in patients with senile dementia of the Alzheimer type. Attempts to treat this disorder with acetylcholine precursors have yielded disappointing results, possibly due to the loss of choline acetyltransferase containing neurons required to convert the exogenous precursor to the active transmitter.

Since muscarinic receptors appear relatively well preserved in Alzheimer-type patients, direct acting cholinergic agonists might prove more effective. Studies carried out thus far with the potent muscarinic agonist, arecoline, have failed to provide unequivocally beneficial results. One difficulty in demonstrating efficacy derives from the inadequacy of available psychometric instruments, especially in situations requiring their repeated administration. An improved test battery has now been devised, and studies of arecoline in Alzheimer's disease will be completed during the coming year.

6. Endorphin Studies

The limited efficacy and potential toxicity of neuroleptics have prompted the search for alternative pharmacologic approaches to the treatment of hyperkinetic extrapyramidal disorders. Recent evidence suggests that fragments of the pituitary hormone β -lipotropin (β -LPH) exert behavioral effects not mediated by opiate receptors. One such compound, des-tyrosine- γ -endorphin (β -LPH₆₂₋₇₇; DT γ E), exhibits neuroleptic-like activity, but (unlike all existing neuroleptics) without directly interfering with dopaminergic transmission. Preliminary and largely uncontrolled clinical observations with DT γ E in Europe suggest the peptide may have antipsychotic activity. In order to evaluate both antidyskinetic and antipsychotic potency, DT γ E was administered subcutaneously to otherwise untreated chronic schizophrenics, some of whom also had tardive dyskinesia. Preliminary results from this double blind, placebo controlled trial suggest no consistent alteration in behavioral, motor, or endocrinologic function. We plan to complete this study, possibly utilizing higher dose levels, and also to extend this work to shorter sequence β -endorphin derivatives (for example, β -LPH₆₆₋₇₇) which are also recently reported to have neuroleptic-like activity.

7. Vasopressin Studies

The search for pharmacologic approaches to the therapy of memory and learning disorders has lead to clinical studies of vasopressin. In the rat, vasopressin and several of its analogues reportedly facilitate complex cognitive tasks including the acquisition and maintenance of learned responses. The recent discovery that vasopressin-containing nerve cell bodies in the supraoptic nucleus project to the hippocampus and other cortical areas may provide some anatomic basis for these observations. The administration of lysine vasopressin has been reported in several small, uncontrolled European studies to improve aspects of attention and memory in individuals with post-traumatic amnesia or senile dementia. Preliminary results of the Section's ongoing, double blind, placebo controlled studies of lysine vasopressin, administered either intranasally or intramuscularly, suggest that some cognitive processes may be improved in Alzheimer's disease patients. Depending on the final outcome, this work may be extended to include a promising new vasopressin analogue, desglycinamide arginine vasopressin (DGAVP), which in the laboratory animal retains the ability to influence learned responses but is relatively free of antidiuretic or pressor activity.

8. Other Peptide Studies

Laboratory investigations of several other naturally occurring peptides or their synthetic analogues have been conducted during the past year. For example, the behavioral effects of various fragments of substance P, including substance P₁₋₇ and substance P₁₋₈ have been observed following systemic or intracranial injection into rats.⁸ These compounds were selected for study because of the possibility that they would, in comparison to natural substance P, penetrate the blood brain barrier better and have a longer central duration of action. In other laboratory investigations the ability of cholecystokinin, which is contained within certain dopamine neurons, to influence various biochemical parameters of presynaptic dopaminergic function, has been evaluated. These studies will continue in an attempt to find alternative pharmacologic approaches to the modification of central dopaminergic function.

9. Positron Emission Tomography Studies

Investigations of regional neuronal activity in patients with various dementing disorders are currently being conducted by means of positron emission tomography following ¹⁸F-2-fluoro-2-deoxyglucose administration. The regional accumulation of the labelled glucose analogue is used as an index of local neuronal function in patients with Alzheimer's disease or Huntington's chorea. Clinically normal individuals, who are at risk for Huntington's disease, serve as controls. Attempts are being made to correlate various aspects of cognitive function under basal conditions as well as during activation by various mental tasks or by drugs which influence higher cortical function. Future plans call for the extension of these studies to patients with hyperkinetic extrapyramidal disorders, such as tardive dyskinesia, Tourettes syndrome, and dystonia musculorum deformans, where no neuropathologic alterations have been found to account for neurologic symptoms.

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C.T. No. 76-N-372
C.T. No. 79-N-115
C.T. No. 79-N-141
C.T. No. 79-N-129
C.T. No. 76-N-122
C.T. No. 74-N-26
C.T. No. 74-N-26A
C.T. No. 79-N-57
C.T. No. 80-N-55
C.T. No. 76-N-117
C.T. No. 76-N-110A
C.T. No. 76-N-270
C.T. No. 78-N-M-156
C.T. No. 77-N-159A
C.T. No. 76-N-23
C.T. No. 78-N-M-138
C.T. No. 76-N-271A
C.T. No. 76-N-121
C.T. No. 76-N-167
C.T. No. 79-N-95
C.T. No. 76-N-166
C.T. No. 76-N-165
C.T. No. 77-N-201
C.T. No. 76-N-174
C.T. No. 80-N-01

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Diagnostic and Therapeutic Reevaluation of Patients with Intractable Epilepsy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. J. Porter Acting Chief, ET NINCDS
Clinical Epilepsy SectionOther: M.E. Newmark Neurologist EB NDP NINCDS
W.T. Theodore Clinical Associate EB NDP NINCDS
R. Long Video Engineer EB NDP NINCDS
H. J. Kupferberg Pharmacologist EB NDP NINCDS

COOPERATING UNITS (if any)

Epilepsy Branch, NDP, NINCDS; Office of Administrative Management,
Clinical Center, NIH

LAB/BRANCH

Experimental Therapeutics Branch

SECTION

Clinical Epilepsy Section

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS☐ (b) HUMAN TISSUES☐ (c) NEITHER☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Despite recent advances in the therapy of epilepsy, many patients, especially those with complex partial seizures are incapacitated by their disorder. We have been investigating improvement of seizure control and reduction of medication side effects through the application of newly developed intensive monitoring techniques including simultaneous video recording of seizures, long-term telemetering of EEGs and frequent determinations of antiepileptic drug concentrations. Patients with very long histories of uncontrolled seizures are admitted for a complete evaluation, including all basic neurologic studies and daily objective toxicity battery. Video recording and long-term telemetered EEGs establish a seizure diagnosis, a concept which has not been adequately emphasized in the management of patients with intractable seizure disorders. Efforts are then made, based on this seizure diagnosis, to "tailor make" a regimen which is appropriate for each patient. This includes use of newer anti-epileptic medications which have decreased side effects in conjunction with blood concentrations which allow maximum therapeutic levels with minimal toxicity.

Project Description:Objectives:

It is the hypothesis of this study that utilization of new techniques of intensive monitoring of patients with intractable seizures can improve clinical control in many patients with refractory seizure problems and can aid in the diagnosis of patients with disorders of unknown type such as psychogenic seizures. The fundamental method of therapy is medical, and modification of therapy will be dependent on collected information from all sources, including detailed history and examination, as well as routine and special laboratory studies, as indicated.

Methods:

Patients with intractable seizures are admitted to the Clinical Center according to the following criteria: 1) Primary consideration will be given to patients with complex partial seizures. Patients with other types of seizure disorders will be considered at a later date. A limited number of patients with suspected psychogenic seizures are also admitted for study. 2) The history of uncontrolled seizures should preferably be a continuous pattern of attacks during the few months prior to admission at the very least, and preferably for many years. 3) Seizure frequency by history must be sufficient to make video monitoring effective. Priority is being given to patients whose seizures occur once or more per day. 4) Patients must be able and willing to cooperate with the entire experimental protocol, including intensive monitoring studies.

Video recordings are made of patients in two separate rooms, which are completely equipped for closed-circuit television studies. Video recordings are made in six-hour periods from 0900 to 1500. Each patient has a minimum of one recording on every new regimen after drug steady state level has been reached. This is compared with the baseline recording for evaluation of the new regimen.

EEG telemetry is performed using either a four or an eight-channel FM-FM transmitter and receiver. The transmitter is worn by the patient and the signal is picked up by a dual diversity antenna and receiver system; the information is integrated into the television format so that a simultaneous display of patient and the electroencephalogram can be obtained.

Blood levels of antiepileptic drugs are determined by gas-liquid chromatography and by immunoassay in the pharmacology laboratory of the Epilepsy Branch.

Initially baseline studies are performed. The seizure frequency and seizure type is characterized by the intensive monitoring techniques, and correlation of this information is obtained with blood levels of the anti-epileptic drugs. The patient subsequently begins new regimens which are based on the seizure diagnosis. Repeat studies of the patient on each new

regimen demonstrates the efficacy of this regimen and whether any change in seizure type or frequency occurs. Each regimen is instituted with the aid of frequent antiepileptic drug levels to assure that each drug is used to maximal benefit with minimal toxicity.

A specific protocol has also been designed to investigate the etiologies of selected patients with epilepsy and progressive neurologic deterioration. This study, which involves a multidisciplinary team of investigators, is capable of analyzing neurologic, electroencephalographic, radiologic, pathologic (including brain biopsy when indicated), metabolic, and virologic data in an attempt to delineate some of the causes of seizure disorders.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

The Clinical Epilepsy Section has become a referral center for patients with intractable seizures, although only a very small number can be accepted into the program. Follow-up studies have been initiated and conducted on all patients who have undergone intensive monitoring to determine the current seizure frequency, changes in social and rehabilitative status, and changes in the physical examination. At reevaluation after two years, 65% of the 46 patients with severe epilepsy still showed improved seizure control compared with preadmission frequency, 63% had reduced drug toxicity, and 41% had improved social adjustment.

Emphasis has been placed on the psychogenic (hysterical seizure). Diagnosis was determined by assessment of four major criteria: deviation of seizures from characteristics of known seizure types, absence of epileptiform activity in the ictal EEG, absence of slowing in the postictal EEG, and relation of seizure frequency to decreasing plasma concentrations of antiepileptic drugs. The clinical characteristics of psychogenic attacks were compared with generalized tonic-clonic and complex partial seizures. Although a 1-year seizure-free period without medication is likely the absolute criterion for psychogenic seizures, intensive monitoring greatly increases the accuracy of the diagnosis, using the criteria developed in this study.

During the past year as part of an approved study of evoked responses of patients with complex partial seizures, normal subjects were evaluated to determine whether the visual evoked response is altered over the hemisphere opposite the nondominant eye. Among 25 normal subjects, the amplitude of the response of the right eye in the right eye dominant group was significantly higher than those with the left eye. A similar trend was noted with left eye dominant subjects, but the difference was not statistically significant. In addition, latencies of the P100 peak measured at O_z were shorter for the dominant eye. These findings demonstrate that the amplitude and latency disparities between dominant and nondominant eyes must be taken into account in any study involving VEPs, in particular studies designed for specific clinical diagnosis or for clinical investigation.

Intensive monitoring techniques of patients with intractable seizures can improve seizure control, decrease medication toxicity, and have a positive effect on the patient's work and social status. The need for reevaluation of patients with uncontrolled seizures is emphasized and the importance of these specialized techniques in obtaining these goals is documented. Furthermore, the Clinical Epilepsy Section continues to be a model for investigators and clinicians interested in setting up such intensive monitoring units throughout the world. The NIH is expected to provide leadership in the technical aspects and scientific uses of intensive monitoring and the Section has continued to provide this.

As experience is gained with intensive monitoring units, it is apparent that much more is needed than demonstration projects which show the usefulness of the technique. Rather the technique is proving to be a powerful investigative tool in a) evolving the proper, objective classification of epileptic seizures, b) controlling seizure types for antiepileptic drug studies, and c) furthering our knowledge of response of various specific seizure types to specific drugs.

Another, long neglected area that has been investigated by intensive monitoring is the psychogenic (hysterical) seizure, a major diagnostic problem that frequently confronts the neurologist. Although these patients tend to be refractory to therapy, causing them to gravitate to specialized centers and making estimates of incidence difficult, several centers using monitoring techniques report a high incidence of patients with such seizures, either with or without associated epileptic attacks. Although the problem is very complex and the patients very heterogeneous, the concepts introduced by studies in the Clinical Epilepsy Section will be useful both to physicians confronted with problem patients and to investigators who wish to increase our understanding in this area.

An important development in the evaluation of patients with intractable seizures is the new technique of positron emission tomography. In patients with intractable partial seizures, early studies have suggested that the local cerebral metabolic rate of glucose is reduced interictally in patients and increased in patients during a seizure. Studies are now underway in patients under a variety of therapies with intractable multifocal seizures, partial seizures, and primary generalized seizures; these patients may also have significant abnormalities in the local cerebral metabolic rate of glucose. This finding may lead to improved localization for surgical therapy for the seizures in selected individuals.

Proposed Course:

1. The Clinical Epilepsy Section continues to make technical advances in intensive monitoring both at the electronic and at the pharmacologic level. There are many further refinements which are indicated, and these have received considerable priority in the Section. Because of the remarkable

heterogeneity of patients with intractable seizures, and because more information is gradually being obtained about the value of intensive monitoring as well as information on various seizure types, patients will continue to be accepted into this study. The program will continue to be active in technology transfer in intensive monitoring, both from technical and scientific standpoints.

2. The efforts of the Clinical Epilepsy Section will be directed toward utilization of the PET scan in evaluation of patients with uncontrolled seizures. Studies on patients with intractable partial seizures, primary generalized seizures, and multifocal seizures are currently underway to determine if changes in therapy, changes the activity of the epileptiform electroencephalographic focus and to determine if potential surgical therapy can be applied.

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C.T. No. 75-N-124

C.T. No. 77-N-195

C.T. No. 78-N-158

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER <div style="text-align: right;">Z01 NS 02318-03 ET</div>												
PERIOD COVERED October 1, 1979 to September 30, 1980														
TITLE OF PROJECT (80 characters or less) Clinical Pharmacology of Antiepileptic Drugs														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: R.J. Porter</td> <td style="width: 33%;">Acting Chief, Clinical Epilepsy Section</td> <td style="width: 33%;">ET NINCDS</td> </tr> <tr> <td>Other: M.E. Newmark</td> <td>Neurologist</td> <td>EB NDP NINCDS</td> </tr> <tr> <td>H. J. Kupferberg</td> <td>Pharmacologist</td> <td>EB NDP NINCDS</td> </tr> <tr> <td>W.T. Theodore</td> <td>Clinical Associate</td> <td>EB NDP NINCDS</td> </tr> </table>			P.I.: R.J. Porter	Acting Chief, Clinical Epilepsy Section	ET NINCDS	Other: M.E. Newmark	Neurologist	EB NDP NINCDS	H. J. Kupferberg	Pharmacologist	EB NDP NINCDS	W.T. Theodore	Clinical Associate	EB NDP NINCDS
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W.T. Theodore	Clinical Associate	EB NDP NINCDS												
COOPERATING UNITS (if any) Epilepsy Branch, NDP, NINCDS														
LAB/BRANCH Experimental Therapeutics Branch														
SECTION Clinical Epilepsy Section														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md. 20205														
TOTAL MANYEARS: <div style="text-align: center;">0.8</div>	PROFESSIONAL: <div style="text-align: center;">0.8</div>	OTHER:												
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) The Clinical Epilepsy Section has been studying the <u>clinical pharmacology</u> of old and new <u>antiepileptic drugs</u> . In spite of the fact that many such medications have been marketed for many years, there is still a great deal to be learned about the proper use of the drugs which are already in our armamentarium. Advances in pharmacology in the past 20 years have allowed the use of new approaches and techniques to gain insight into the proper use of our currently available drugs. In addition, the Clinical Epilepsy Section has been involved in <u>clinical trials</u> of new antiepileptic drugs, such as sodium valproate which was recently marketed in the United States. The pharmacologic evaluation of these drugs is coupled with efficacy studies which are carried out by <u>intensive monitoring techniques</u> including videotape analysis of epileptic seizures with simultaneous electroencephalographic recording and long-term telemetered EEG recording. The daily determination of antiepileptic drug levels is an integral part of the on-going studies. Recent emphasis has been on the effect of food on the absorption of antiepileptic drugs, and on mechanisms for valproic acid-phenobarbital interaction.														

Project Description:Objectives:

This project includes a large number of independent pharmacologic studies in the investigation of the clinical pharmacology of antiepileptic drugs. The object of each study is different, but each may include obtaining such pharmacologic data as (1) single dose plasma half-lives, (2) relative plasma levels of parent drugs and metabolites with chronic administration, (3) relationships of the efficacy of parent drugs and/or metabolite to plasma levels, (4) efficacy of various compounds against different seizure types as correlated with intensive monitoring techniques, (5) evaluation of efficacy and toxicity of new antiepileptic agents, such as sodium valproate, (6) measurement of rate of biotransformation of various antiepileptic medications, and (7) determination of the clinical consequences of withdrawal of antiepileptic drugs.

Methods:

Patients with uncontrolled seizures, especially complex partial seizures, are accepted for study. Such patients usually have a detailed seizure calendar available prior to entering the study, and enter a week-long period of baseline determination of seizure frequency and blood levels of antiepileptic drugs while in the hospital. Each pharmacologic protocol varies, but all require modification of the antiepileptic regimen and addition of the medication under study. This may be done in single dose or chronic administration studies depending upon the particular protocol in question. As a rule, plasma levels are drawn at least daily, and on occasion, much more frequently for specific studies. Following the completion of the pharmacologic protocol, the patient is placed on a regimen which is best suited for the seizure type which has been identified by videotape/telemetered EEG analysis. This regimen is stabilized prior to discharge of the patient.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) Although interest has recently developed on the effect of food on absorption of a wide variety of medications, little evidence has thus far accumulated on the effect of food on antiepileptic drugs. Initial studies performed on volunteers have suggested that food may affect the absorption of carbamazepine. In the studies conducted by the Clinical Epilepsy Section the effect of taking medication with food and without food has been analyzed in seven patients. Steady state levels obtained over a two-week therapy, as well as after single administration, have been analyzed. Although steady state levels do not appear to change, food does appear to affect the rate of absorption of carbamazepine. These preliminary findings suggest that food may be an important factor in the absorption of medications in individual patients, and that careful drug administration may greatly influence the total dose of drug that a patient can tolerate.

Recognition of their subtle but significant toxicity has made the need for sedative-hypnotic antiepileptic drugs less certain, but their removal is often thought to be difficult and dangerous. Barbiturates and benzodiazepines were completely withdrawn from 66 patients with intractable epilepsy (39 inpatients and 27 outpatients). After 6 months of outpatient follow-up, 59 patients were still on a nonsedative regimen: 47 showed improvement in either toxicity or seizure control or both. This study shows that sedative drugs are not necessary for optimal seizure control, even in intractable epilepsy, and that they may be safely withdrawn.

2) Valproic acid (VPA, Depakene) was recently marketed in the U.S. for the treatment of epileptic seizures. It has been reported to cause a significant elevation of plasma phenobarbital levels. The mechanism of this drug-drug interaction was not previously elucidated. Three possible mechanisms of interaction were investigated: (A) inhibition of phenobarbital metabolism, (B) increased renal reabsorption of phenobarbital via urinary pH effect, and (C) change in volume of distribution. Valproic acid was added to the phenobarbital regimens. Pharmacokinetic parameters were obtained by determining the area under the curve of a single pulse dose of [1,3-¹⁵N, 2-¹³C]phenobarbital before and after the administration of valproic acid. Plasma levels of labelled phenobarbital were determined by gas chromatography/mass spectrometry. Urinary pH, urinary phenobarbital levels, and urinary p-OHPB levels (free and total) were also quantitated. The volume of distribution and urinary pH did not change during the entire experimental period, thus eliminating mechanisms (B) and (C). Plasma phenobarbital increased approximately 25%, with a concomitant decrease in total body phenobarbital clearance and decrease in excretion of total p-OHPB. Therefore, these results support mechanism (A).

More recently, we have studied the interaction of valproic acid with acetaminophen in 3 patients. Both agents are partially metabolized by conjugation with glucuronide. In these studies, acetaminophen metabolism was not affected by chronic therapy with valproic acid, thereby demonstrating that the conjugation for these two agents is different, and strongly suggesting that the inhibition of phenobarbital metabolism by valproic acid is at the hydroxylation reaction.

3) A planned project for the Clinical Epilepsy Section will be directed toward differential phenytoin metabolism. It has been noted that different racial groups and nationalities may have different pharmacokinetic handling of a wide assortment of medications. Since phenytoin is very widely used internationally as an antiepileptic drug, the determination of differences in the pharmacokinetics of phenytoin among different nationalities is quite important. The Clinical Epilepsy Section has initiated studies with stable labelled isotopes which will allow careful and accurate identification of the metabolism of phenytoin in patients without having to withdraw the patients from this medicine. A pilot study for a WHO protocol is planned for the NIH using the stable labelled isotope to evaluate the adequacy of the proposed protocol; this will be followed by the international study.

4) The effect of food on absorption of antiepileptic drugs is a pilot project which may eventually lead to quite significant findings. Since food has been known to affect the absorption of other drugs, it is reasonable to expect that it may also affect absorption of at least some of the antiepileptic drugs. Because of the occasionally narrow ratio between toxicity and therapeutic levels, the effect of food on efficacy and toxicity may be quite significant for an individual patient. In our initial studies where the absorption of carbamazepine acutely was affected in several patients, the effect of food may eventually have quite profound importance for dosage schedules, instructions to patients, and eventual maximum amount of medication which might be given. This is especially important for severely affected patients because of the need for maximal doses of medications to obtain optimal control.

5) Most of the drugs currently employed in the treatment of epileptic disorders have a relatively narrow therapeutic ratio and when used in conjunction with other antiepileptics or other drugs, they frequently become involved in drug-drug interactions. The propensity of these agents for drug-drug interaction complicates effective and safe management of seizures. A new antiepileptic drug is structurally unrelated to conventional anticonvulsant drugs. It has a broad spectrum of antiepileptic action and is remarkably free of toxic side effects. However, when valproic acid is administered in conjunction with other antiepileptics, most notably phenobarbital, it potentiates side effects of the latter. This results in increased sedation, and even coma in some patients. It is important to understand the mechanism of this interaction, so that the physician can be aware of which organ systems are involved and what can most effectively be done to overcome this problem. For the future of antiepileptic drug development, this kind of pharmacologic information is valuable in the consideration of the design of new drugs. In general, it has been well demonstrated that the more we understand about the pharmacology and mechanism of actions of drugs, the more effectively we can utilize these to the benefit of the patients involved.

6) The use of stable labelled isotopes is a very important pharmacologic technique which the Clinical Epilepsy Section is currently utilizing. With the use of stable labelled isotopes, the pharmacokinetics of drugs may be studied in patients without having to withdraw the patient from the drug. This technique, therefore, allows a more accurate appraisal of the pharmacokinetics of the drug in a patient under realistic practices without toxicity problems such as those associated with radioactive tracers. In the pilot study which is planned, differences in metabolism among different racial groups will be studied. This study may be quite important on an international scale as it may help explain differences in efficacy and toxicity among the various ethnic groups.

Proposed Course:

1) Future studies will attempt to elucidate further such variables as absorption, distribution, metabolism, excretion, drug interaction, efficacy, toxicity, withdrawal.

2) A planned project for the Clinical Epilepsy Section will be directed toward the study of differential phenytoin metabolism in different races. A study for a WHO protocol is planned for the NIH using a stable labelled isotope to evaluate the adequacy of a pilot protocol; this will be followed by an international study.

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C.T. No. 76-N-344
C.T. No. 77-N-92
C.T. No. 78-N-171
C.T. No. 79-N-04

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02263-04 ET																				
PERIOD COVERED <p style="text-align: center;">October 1, 1979 to September 30, 1980</p>																						
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Biochemical and Pharmacological Studies of Dopamine Receptors</p>																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">J.W. Kebabian</td> <td style="width: 40%;">Head, Biochemical Neuropharmacology Unit</td> <td style="width: 10%;">ET NINCDS</td> </tr> <tr> <td rowspan="5">Other:</td> <td>T. E. Cote</td> <td>Staff Fellow</td> <td>ET NINCDS</td> </tr> <tr> <td>M. Munemura</td> <td>Visiting Fellow</td> <td>ET NINCDS</td> </tr> <tr> <td>K. Tsuruta</td> <td>Visiting Fellow</td> <td>ET NINCDS</td> </tr> <tr> <td>R. Eskay</td> <td>Senior Staff Fellow</td> <td>LCS NIMH</td> </tr> <tr> <td>R. Long</td> <td>Biologist</td> <td>LCS NIMH</td> </tr> </table>			PI:	J.W. Kebabian	Head, Biochemical Neuropharmacology Unit	ET NINCDS	Other:	T. E. Cote	Staff Fellow	ET NINCDS	M. Munemura	Visiting Fellow	ET NINCDS	K. Tsuruta	Visiting Fellow	ET NINCDS	R. Eskay	Senior Staff Fellow	LCS NIMH	R. Long	Biologist	LCS NIMH
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COOPERATING UNITS (if any) <p style="text-align: center;">Laboratory of Clinical Science, NIMH</p>																						
LAB/BRANCH <p style="text-align: center;">Experimental Therapeutics Branch</p>																						
SECTION <p style="text-align: center;">Biochemical Neuropharmacology Unit</p>																						
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Md. 20205</p>																						
TOTAL MANYEARS: <p style="text-align: center;">5.3</p>	PROFESSIONAL: <p style="text-align: center;">4.3</p>	OTHER: <p style="text-align: center;">1.0</p>																				
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SUMMARY OF WORK (200 words or less - underline keywords) <p> This project identifies and characterizes biochemical mechanisms for <u>dopamine receptors</u>. An understanding of the biochemical phenomena contributing to the activity of dopamine receptors will identify possible mechanisms of action for drugs used to treat <u>Parkinson's disease</u>, <u>endocrine disorders</u>, and <u>psychiatric disorders</u>. Among the topics studied in the current fiscal year are: 1) <u>in vitro</u> characterization of the D-2 receptor in the intermediate lobe of the rat pituitary gland; 2) identification of the kinetic and biochemical consequences of stimulation of the D-2 receptor; and 3) identification of the role GTP in the functioning of the D-2 receptor. </p>																						

Project Description

Objectives:

1. Characterize pharmacological properties of the D-2 receptor in the intermediate lobe of rat pituitary gland
2. Characterize the biochemical and kinetic basis for the physiological actions of the D-2 receptor
3. Study the involvement of GTP in the functioning the D-2 receptor.

Methods:

The intermediate lobe of the rat pituitary gland contains a beta-adrenoceptor and a D-2 dopamine receptor. The presence of a beta-adrenoceptor inferred from the observations that beta-adrenergic agonists enhance the release of alpha-melanocyte stimulating hormone (α MSH), enhance the formation of cyclic AMP (cAMP), stimulate adenylate cyclase activity, and compete with (125 I)-hydroxybenzypendolol for specific binding sites. Similarly, the presence of a dopamine receptor can be inferred because dopaminergic agonists inhibit the spontaneous release of α MSH; and inhibit the responsiveness of the beta-adrenoceptor.

Thus, the procedures used in this study are: determination of α MSH; determination of cyclic AMP; determination of adenylate cyclase activity; and (125 I)-HYP binding determinations. The assays are performed upon cell-free homogenates of intermediate lobe tissue or upon preparations of dispersed cells of the intermediate lobe.

Major Findings (during Fiscal Year 1980) and their Significance to Biomedical Research:

When the subdivision of dopamine receptors into two categories, designated D-1 and D-2 was first proposed, the mammothrophs of the anterior pituitary gland, the cells which synthesize and secrete prolactin, provided the best example of a cell which possessed a D-2 receptor. However, direct investigation of the biochemistry of the dopamine receptor on the mammothrophs was not possible because the heterogeneous composition of the anterior pituitary gland precluded the preparation of a homogeneous population of mammothrophs. In order to study the D-2 receptor in a pure population of cells, recent experiments (performed in collaboration with Dr. Eskay of NIMH) have investigated the biochemistry and physiology of the D-2 receptor in the intermediate lobe of the rat pituitary gland. The intermediate lobe is a homogeneous tissue which synthesizes and secretes alpha-melanocyte stimulating hormone (alpha-MSH) and several other peptide hormones derived from a single precursor molecule. A beta-adrenoceptor linked to adenylate cyclase occurs upon the parenchymal cells of the intermediate lobe. Stimulation of this beta-adrenoceptor enhances the formation of cyclic AMP and the release of alpha-MSH.

A dopamine receptor also occurs on the parenchymal cells of the intermediate lobe. Stimulation of this dopamine receptor diminishes the spontaneous release of α -MSH. Furthermore, stimulation of the dopamine receptor decreases the consequences of activation (i.e., the "responsiveness") of the beta-adrenoceptor. The pharmacological properties of the dopamine receptor in the IL were deduced from the ability of dopamine, dopaminergic agonists or dopaminergic antagonists either to inhibit the spontaneous release of α -MSH or to alter the responsiveness of the IL to beta-adrenergic stimulation.

The interaction between the D-2 receptor and the β -adrenoceptor is amenable to biochemical investigation because this interaction can be studied either in experiments utilizing preparations of intact IL cells or in experiments utilizing cell-free homogenates of the IL. In intact IL cell experiments, the responsiveness of the beta-adrenoceptor is inferred from the ability of L-isoproterenol, a pure beta-adrenergic agonist either to enhance the release of α -MSH or to enhance the formation of cyclic AMP. Stimulation of the dopamine receptor diminishes either response to simultaneous stimulation of the beta-adrenoceptor. In cell free experiments, the responsiveness of the beta-adrenoceptor is inferred from the ability of beta-adrenergic agonists to enhance the formation of cyclic AMP by the enzyme adenylate cyclase, thus, the ability of the D-2 receptor to diminish the responsiveness of the beta-adrenoceptor survives homogenization and can be demonstrated in several types of experiments.

The inhibitory effect of the D-2 receptor predominates over the stimulatory effect of the beta-adrenoceptor. In cell free experiments dopamine can be shown to interact with the beta-adrenoceptor and the D-2 receptor. Thus, dopamine enhances adenylate cyclase activity in cell free homogenates of the IL. This beta-adrenergic effect of dopamine is potentiated by the dopamine antagonist fluphenazine; but blocked by the beta-antagonist propranolol. However, in similar experiments, apomorphine does not interact with the beta-adrenoceptor (either as an agonist or an antagonist). Apomorphine is the "agonist of choice" for biochemical investigations of the dopamine receptor because apomorphine decreases the maximal, L-isoproterenol-induced enhancement of adenylate cyclase without altering the molar potency of the beta-adrenergic agonist. Kinetic analysis of the dose-dependent decrease in the response to L-isoproterenol shows that apomorphine decreases the response to L-isoproterenol in a non-competitive fashion. This action of apomorphine contrasts with the blockade of the beta-adrenoceptor with propranolol; propranolol competes with L-isoproterenol for occupancy of the beta-adrenoceptor. The dopamine receptor can be assigned to the D-2 category on the basis of the following criteria: 1) stimulation of the dopamine receptor does not result in enhancement of adenylate cyclase activity or an accumulation of cAMP; 2) the dopaminergic ergots, lergotril and bromocriptine, mimic the inhibitory effect of dopamine and; 3) metoclopramide and sulpiride, substituted benzamides, block the inhibitory effect of dopamine.

The guanyl nucleotide, GTP, is required to obtain the maximal response to the beta-adrenergic agonists, L-isoproterenol. Thus, GTP potentiates, in a dose-dependent manner, the L-isoproterenol-stimulated adenylate cyclase activity. This effect of GTP is diminished by simultaneous stimulation of the D-2 receptor; apomorphine decreases the ability of GTP to enhance L-isoproterenol-stimulated adenylate cyclase activity. This inhibitory effect of apomorphine is reversed by the dopaminergic antagonist, fluphenazine.

The following "working hypothesis" summarizes our thinking about the organization of the beta-adrenoceptor and the D-2 receptor; stimulation of the D-2 receptor decreases the ability of GTP to enhance L-isoproterenol-stimulated adenylate cyclase activity and thereby causes a noncompetitive decrease in the responsiveness of the beta-adrenoceptor. A dopamine antagonist, which blocks the D-2 receptor, reduces the inhibitory constraint upon the action of GTP and thereby potentiates the effect of a beta-adrenergic agonist. This "working hypothesis" also applies to the intact cells of the intermediate lobe because L-norepinephrine enhances the formation of cyclic AMP by these cells only in the presence of a dopamine antagonist (Munemura, Cote, Tsuruta, Eskay and Keabian, unpublished).

Proposed Course:

In the coming year, research will continue to focus on the effects of dopamine in the intermediate lobe. Dopamine, acting upon the D-2 receptor, has three known physiological effects in this tissue: 1) inhibition of basal release of alpha-MSH; 2) inhibition of spontaneous electrical activity and 3) decreasing responsiveness of beta-adrenoceptor. We will attempt to determine if the three events are interrelated by attempting to "factor out" each response. Thus, tetrodotoxin will block sodium channels and abolish electrical activity; we can then test the effects of dopamine upon α MSH release and also responsiveness of the beta-adrenoceptor in the absence of electrical activity. Likewise we can test depolarizing agents, such as K^+ ions, which will mimic the spontaneous electrical activity. The short term goal is to define and identify the interrelationship between the different physiological processes which dopamine regulates.

Publications:

- Cote, T., Munemura, M. and Keabian, J.W.: Lisuride hydrogen maleate: An ergoline with beta-adrenergic antagonist activity. Eur. J. Pharmacol. 59: 303-306, 1979.
- Keabian, J.W., Chen, T.C. and Cote, T.E.: Endogeneous guanyl nucleotides: Components of the striatum which confer dopamine-sensitivity to adenylate cyclase. Communications in Psychopharmacology 3: 421-428, 1979.

- Cote, T.E., Chen, T.C. and Keboabian, J.W.: Guanosine triphosphate: An endogenous compound in the rabbit cerebellar cortex which couples the beta-adrenergic receptor to adenylate cyclase. Brain Res. 181: 127-138, 1980.
- Chen, T.C., Cote, T.E. and Keboabian, J.W.: Endogenous components of the striatum confer dopamine-sensitivity upon adenylate cyclase activity: The role of endogenous guanyl nucleotides. Brain Res. 181: 139-149, 1980.
- Munemura, M., Eskay, R.L., and Keboabian, J.W.: Release of alpha-melanocyte stimulating hormone from dispersed cells of the intermediate lobe of the rat pituitary gland: Involvement of catecholamines and cyclic AMP. Endocrinology 106: 1795-1803, 1980.
- Cote, T.E., Munemura, M., Eskay, R.L., and Keboabian, J.W.: Biochemical identification of the beta-adrenoceptor and evidence for the involvement of a cyclic AMP system in the beta-adrenergic induced release of alpha-melanocyte stimulating hormone in the intermediate lobe of the rat pituitary gland. Endocrinology 107: 108-116, 1980.
- Keboabian, J.W. and Zatz, M.: Adaptive properties of adrenoceptors. Cell Surface Reviews. 1980. In press.
- Keboabian, J.W. and Cote, T.E.: Dopamine receptors and cyclic AMP: A decade of progress. Trends in Pharmacological Sciences. 1980. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02139-06 ET
PERIOD COVERED October 1, 1979 through September 30, 1980		
TITLE OF PROJECT (80 characters or less) Pharmacology and Physiology of Central Neurotransmitters		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J.R. Walters Head, Physiological Neuropharmacology Unit, ETB, NINCDS Others: B.L. Waszczak Staff Fellow ETB NINCDS D. Bergstrom Staff Fellow ETB NINCDS R. Hruska Staff Fellow NTS/ODIR NINCDS		
COOPERATING UNITS (if any) NTS, ODIR, NINCDS		
LAB/BRANCH Experimental Therapeutics Branch		
SECTION Physiological Neuropharmacology Unit		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.7	PROFESSIONAL: 3	OTHER: 1.7
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SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this project is to improve our understanding of ways in which drugs may alter centrally mediated <u>neurotransmission</u> and to develop better <u>pharmacotherapies</u> for <u>neurological disorders</u> . Topics currently under investigation include: (1) the potential role of dopamine <u>autoreceptors</u> and the <u>striatonigral "feedback loop"</u> in regulation of dopamine activity and mediation of dopamine <u>agonist effects</u> ; (2) the role of <u>GABA</u> afferents to the <u>substantia nigra</u> and consequences of <u>GABA</u> agonist administration (3) the role of <u>cholecystokinin (CCK)</u> in dopaminergic function; (4) effects of <u>neurotransmitter agonists and antagonists</u> on the activity of <u>globus pallidus neurons</u> and (5) mechanisms involved in endogenous regulation of <u>glutamic acid decarboxylase</u> activity.		

Project Description:Objectives:

This project involves investigation of the role of specific neurotransmitters in regulating neuronal activity in extrapyramidal systems. The long-range goal is to establish improved pharmacological treatment to compensate for apparent neuronal degeneration and dysfunctions which occur in a variety of neurological disorders involving the basal ganglia and substantia nigra regions. Parkinsonism, for example, is associated with loss of dopamine-containing nigrostriatal neurons. Profound loss of GABA, substance P and in some cases, acetylcholine-containing striatal and pallidal neurons attends Huntington's chorea. The extrapyramidal brain regions may be significant in the etiology of many other types of neurological and psychiatric disorders as well, since many drugs which have significant positive or negative effects on these disease states, also have significant effects on neuronal function in the basal ganglia and substantia nigra. Thus, current studies of this Unit are dedicated to understanding the normal processes regulating information flow in these brain regions and the various ways in which pharmacological agents may interact with these processes.

Methods:

These studies utilize: (a) determination of single-unit activity of identified brain cells in anesthetized rats, some with neurotoxin-induced lesions of specific brain regions, (b) determination of single-unit activity in identified brain regions in rats which are locally anesthetized, paralyzed and artificially respired, (c) investigation of effects of microiontophoresed drugs or neurotransmitter substances on single-unit activity, (d) estimation of brain lesion or drug-induced changes in levels of dopamine and glutamic acid decarboxylase, and in the apparent *in vivo* synthesis rate of dopamine by measurement of dopamine precursor and metabolite levels in brain homogenates, and (e) analysis of enzyme activity in synaptosomes, homogenates or precipitates of brain tissue obtained from decapitated rats or rats sacrificed with a "brain blowing" device.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

In order to obtain a better understanding of factors regulating neuronal activity in the substantia nigra and the consequences of pharmacological intervention, our current investigations have focused on 4 areas: (1) the potential role of dopamine autoreceptors and the striatonigral "feedback loop" in regulation of dopamine activity and mediation of dopamine agonist effects; (2) the role of the GABA afferents to the substantia nigra and consequences of GABA agonist administration; (3) the role of cholecystikinin (CCK) in dopaminergic function; (4) effects of neurotransmitter agonists and antagonists on the activity of globus pallidus neurons; and (5) factors involved in regulation of GABA synthesis.

(1) Dopamine Autoreceptors and the Striatonigral "Feedback Loop" in Regulation of Dopamine Activity: Effects of Dopamine Agonists

Early hypothesis about the regulation of the nigrostriatal dopamine pathway by a striatonigral "feedback loop" predicted that the activity of the nigral dopamine neurons would be inhibited by dopamine receptor stimulating agents interacting with postsynaptic dopamine receptors in the striatum. It has since been shown that drugs which increase the stimulation of dopamine receptors, either by increasing dopamine levels (L-dopa), by increasing the release of dopamine (amphetamine), or by mimicking the action of dopamine (apomorphine) do depress the activity of substantia nigra pars compacta dopamine cells when administered systemically. Similarly, we have found that lergotril and lisuride, ergot derivatives with dopamine agonist properties and antiparkinson efficacy, also inhibit the activity of dopamine neurons. However, the role of the striatonigral pathway as a "feedback loop", mediating the effects of these agonists and regulating dopamine cell activity began to be questioned when iontophoretic studies suggested that dopamine agonists may directly inhibit the activity of dopamine neurons by acting at dopamine receptors on the dopamine cell bodies (autoreceptors). Accordingly, we have been investigating the relative contributions of the dopamine autoreceptors and the striatonigral feedback loop to the effects of systemically administered dopamine agonists by the use of kainic acid injections into the rat neostriatum.

Kainic acid is thought to lesion neuronal cell bodies, leaving incoming axons intact. Following a neostriatal kainic acid injection, the output pathway from the neostriatum, including the striatonigral fibers believed to function as part of a feedback loop from the striatum to the substantia nigra, is destroyed, while the dopamine cells projecting to the neostriatum remain intact. We found that systemically administered apomorphine still effectively inhibits the activity of the dopamine cells after kainic acid-induced lesion of the striatonigral pathway. Lisuride, however, which is normally as potent as apomorphine at inhibiting the activity of dopamine cells, was less effective in lowering dopamine cell firing rates in animals with unilateral striatal lesions. We concluded from these observations that lisuride was less effective than apomorphine at stimulating dopamine receptors on the dopamine cell body and was probably acting, at least in part, to inhibit dopamine cell activity, through dopamine receptors in the striatum associated with the striatonigral feedback loop.

More recently, however, we have made the surprising observation that the effect of lisuride on dopamine firing rates is also attenuated on the side opposite the lesion in rats which have received unilateral kainic acid injections in the striatum. Since there is no obvious cell loss on the side of the brain contralateral to the kainic acid injection, and no known direct crossover of the brain's two striatonigral pathways, it is unclear why an ipsilateral striatal injection of kainic acid would alter the response of the contralateral dopamine neurons to lisuride treatment. Some part of the brain affected by the unilateral kainic acid injections, or some other consequence

of the injection, appears able to modify the effects of lisuride on dopamine cell activity on the contralateral, intact side of the brain. This finding has interesting implications with regard to the mechanism of action of the ergots and the mechanisms involved in regulation of dopaminergic activity. Since lisuride's effects on dopamine activity are different from those of apomorphine in animals with unilateral striatal lesions, yet quite similar to those of apomorphine in normal rats, it would appear that two dopamine agonist-type drugs with markedly similar potencies can inhibit dopamine cell firing by different mechanisms. One difference between apomorphine and many of the ergots, including lisuride, is that the ergots interact with other neurotransmitter systems to a greater extent than does apomorphine. Lisuride especially, is a very potent serotonin agonist and also has norepinephrine antagonist properties. We have wondered whether some effect of lisuride on the dopamine system, mediated through either the serotonin or norepinephrine systems, was affected by the kainic acid lesions. To date, we have observed that the unilateral striatal kainic acid injections do not alter the ability of lisuride to inhibit the firing of the serotonin neurons in the midbrain raphe. This suggests that the raphe cells are not mediating the kainic acid-induced change in lisuride's dopaminergic effects. It also indicates that the kainic acid injections are not affecting the metabolism of lisuride and its distribution to the brain.

Our observations also imply that it will be necessary to reevaluate studies utilizing the kainic acid intrastratial injection technique to examine the role of the striatonigral pathway. A change in dopaminergic function cannot be attributed solely to loss of the ipsilateral striatonigral pathway if it is not demonstrated that dopaminergic function is unaffected on the contralateral side where the striatonigral pathway is intact. For example, it has been reported in the literature that the effects of one other drug, the indirect-acting dopamine agonist, amphetamine, are attenuated ipsilaterally by unilateral intrastratial kainic acid injections. We are in the process of repeating this study, since the original investigation concluded that the attenuation observed in the kainic acid lesioned rats was due to the loss of ipsilateral striatonigral innervation and did not determine whether the effects of amphetamine were unchanged on the contralateral side where the striatonigral pathway was intact.

(2) The Role of GABA and Effects of GABAmimetics in the Substantia Nigra and A10 Dopaminergic Region

The striatal afferents to the substantia nigra are, in part, GABA-containing processes. This has led to the expectation that GABAergic mechanisms are involved in the striatonigral "feedback" regulation of dopaminergic activity. However, just as the apomorphine studies raised some doubt about the significance of the "feedback loop" in regulation of dopamine activity, our ongoing investigations with GABA agonists have continued to raise doubts about how much potential the dopamine neurons have for significant direct modulation by GABAergic processes. We have previously demonstrated that the GABA agonist, muscimol, excites the dopamine cells of the substantia nigra pars compacta when administered systemically and only weakly inhibits these dopamine cells

when iontophoresed onto the dopamine cell body from a 5-barrel iontophoresing and recording micropipette. On the other hand, muscimol does effectively inhibit the single unit activity of cells in the substantia nigra when administered either systemically or iontophoretically. These results suggested that if the dopamine cells are regulated by GABAergic neurons, specifically the striatonigral GABA pathway, this interaction is predominantly indirect and may involve a second inhibitory neuron within the nigra. Alternatively, it may be possible that muscimol (or GABA) exerts an unconventional excitatory response at the level of the dopamine dendrites (but not the cell bodies), as has been described for some hippocampal cells.

In order to confirm these observations we have been examining the effects of a second GABA agonist, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol (THIP). This compound is a rigid, bicyclic isoxazole derivative of GABA and has recently been distinguished both from GABA and muscimol by virtue of its greater relative specificity for inhibiting 3^H -GABA binding than for stimulating benzodiazepine binding. In addition, it has been suggested that THIP might be more suited than muscimol for use as an in vivo GABAmimetic agent because it appears to penetrate the blood-brain barrier more readily than muscimol, it may be more stable in vivo and it may be less toxic than muscimol after systemic administration. We were interested in comparing the potency of THIP to those of GABA and muscimol and in determining whether the somewhat unexpected effects of muscimol on dopamine activity in the nigra would be seen with another GABA agonist.

Since we had previously determined that the pars reticulata neurons of the substantia nigra could be completely inhibited by both systemic and iontophoretic administration of muscimol and since it appears they may be a termination site of a major GABAergic projection from the striatum and globus pallidus, we felt the inhibitory response of the nigral pars reticulata cells to GABAmimetic agents could be viewed as a physiologically relevant test system for studying the GABAmimetic actions of THIP in the CNS and for comparing the potencies of THIP, GABA and muscimol. The effects of iontophoretic administration of THIP were found to be similar to those of muscimol and GABA for both inhibiting reticulata cell firing and 3^H -GABA binding with an order or potency of muscimol < GABA < THIP. The magnitude of the differences between their potencies in iontophoresis studies closely paralleled their relative potencies in binding studies (performed in collaboration with Dr. R. Hruska, Project No. Z01 NS 02264-04 NTS/ODIR), with muscimol approximately 3 times more potent than GABA and between 25 and 40 times more potent than THIP. After systemic i.v. administration, however, muscimol was only 3 times more potent than THIP at inhibiting reticulata cell firing, possibly because THIP more readily passes the blood-brain barrier. This observation together with the possibilities that THIP might be less toxic than muscimol and may interact selectively at one type of GABA site in the brain, suggest that THIP might be a useful drug for exploring the therapeutic potential of GABAmimetic agents in the clinic.

We have also investigated the effects of systemic and iontophoretic administration of THIP upon the dopamine neurons in the substantia nigra. Although THIP was less potent than muscimol, it had stimulatory effects on dopamine cell

activity when administered systemically which were qualitatively similar to those seen with muscimol. These were not reversed by picrotoxin administration. When administered iontophoretically, THIP, like muscimol, weakly inhibited dopamine cell activity. Thus, these findings confirm our previous observations. Moreover, since THIP is excreted largely unchanged and appears to penetrate the blood-brain barrier more readily than muscimol, the results argue against the possibility that the stimulatory effect of muscimol on dopamine cell activity were due to a metabolite of muscimol and not related to its GABA-mimetic actions in the CNS.

The observation that muscimol and THIP did not inhibit the activity of the substantia nigra dopamine cells when administered systemically raised the question of how the drugs might affect the adjacent population of dopamine neurons, the mesolimbic A10 dopamine cells of the ventral tegmental area (VTA). Although evidence is less extensive and the anatomy of the system is different a GABAergic feedback pathway has been postulated to also regulate the activity of these neurons. Anatomical studies have demonstrated a projection from the nucleus accumbens to the VTA, and some evidence suggests that the projection contains GABA neurons. In view of the presumed sensitivity of A10 neurons to endogenous GABA, their responses to GABA-mimetic drugs were studied. It was found that increasing i.v. doses of either muscimol or THIP caused dose-related increases in extracellular, single unit activity of the A10 dopaminergic neurons in the VTA. Muscimol, stimulated firing at doses approximately 15-20 times lower than the doses of THIP required to elicit equivalent excitatory effects. The maximum stimulation was approximately 150-140% of the baseline firing rate for muscimol and THIP, respectively. The similar responses of the two dopamine cell populations suggest that common neuronal mechanisms may mediate them. These findings, therefore, contribute to the notion that the nigrostriatal (A9) and the mesolimbic (A10) dopamine systems may be functionally as well as anatomically parallel and analogous. They also suggest that the systemically administered GABA agonists influence other neurons or pathways whose effects upon dopamine cell firing are more predominant than the direct effects of these agonists at the dopamine cell body. These observations have important clinical ramifications. Since increased dopamine-mediated transmission in the mesolimbic system has been implicated in the etiology of schizophrenia, attention has focused on the potential therapeutic usefulness of GABA agonists in attenuating A10 dopamine cell firing. Our results predict that these drugs might actually increase A10 cell activity and ultimately worsen rather improve symptoms in schizophrenia; an effect consistent with the clinical observation by the Pharmacology Section (Project No. Z01 NS 02265-04 ET) that muscimol not only failed to improve, but, in many cases, increased psychosis scores in schizophrenic patients.

We have also continued to explore the question of whether supersensitivity to GABA develops in the CNS at sites postsynaptic to a lesioned GABA pathway. Intra-striatal injections of kainic acid have been utilized to destroy the striatonigral GABA pathway. We previously determined that 2 to 3 weeks after the striatal lesions there are no significant changes in the sensitivity of the cells in the pars reticulata of the substantia nigra to systemically administered muscimol. More recently we have examined the sensitivity of the

reticulata cells to iontophoresed GABA. In these studies, we have found a small, but apparently significant increase in the ability of GABA to depress unit activity in the lesioned animals. However, since the reticulata cells in lesioned animals did not demonstrate supersensitivity to i.v. muscimol, their increased response to iontophoresed GABA may not be predictive of physiologically important or clinically significant changes in reticulata cell function in diseases such as Huntington's where striatonigral GABA pathways degenerate. This observation is consistent with the finding that muscimol has not generally proved helpful in the treatment of Huntington's chorea.

(3) The Role of Cholecystokinin (CCK) in Dopamine Function

Recent studies have demonstrated that the peptide, cholecystokinin, is stored in some dopamine neurons, most predominantly in the dopamine cells of the ventral tegmental area which project to the accumbens. We have begun to investigate the possible role of this peptide by examining its effects on the single unit activity of the dopamine neurons in the ventral tegmental area. Preliminary studies indicated that systemic administration of CCK causes some increases in dopamine unit activity and attenuates the inhibitory effects of amphetamine on dopamine firing rates. In the course of these studies, however, we found that the CCK we were using was mostly something other than the CCK octapeptide we believed we had purchased. Thus, we have postponed the iontophoretic studies we had planned and feel we must reexamine our initial results once we obtain a sufficient quantity of bonafide CCK.

(4) Effects of Neurotransmitter Agonists and Antagonists on the Activity of Globus Pallidus Neurons

This is the first of a series of studies in which we intend to explore the acute and chronic effects of systemic administration of dopamine agonists and antagonists in brain regions in which a dopaminergic innervation appears likely to have a significant direct or indirect effect. Many of our studies have examined how agonists and antagonists of dopamine and other transmitters affect the firing rates of the nigrostriatal dopamine neurons. Changes in the activity of striatal neurons induced by some of these drugs have been described by others. Currently, we are examining the effects of systemic administration of apomorphine, amphetamine and haloperidol on the single unit activity of the cells in the globus pallidus, an area receiving a major input from the striatum. Dopamine neurons projecting to the subthalamic nucleus may also indirectly affect the activity of this brain region which corresponds to the external pallidal segment in humans.

In paralyzed, artificially respired and locally anesthetized rats, tonically firing pallidal units were slightly but significantly inhibited by haloperidol administration. With lower doses of apomorphine we thought we might see changes like those caused by haloperidol, since small doses of apomorphine are thought to preferentially affect dopamine autoreceptors, inhibiting the dopamine neurons without significantly stimulating the postsynaptic dopamine receptors. No significant changes in pallidal activity were noted, however.

Doses of apomorphine larger than those required to alter dopamine cell activity did increase the activity of 1/3 of the pallidal units recorded. The remaining cells were not affected by apomorphine. Curiously, we found that haloperidol, administered to animals pretreated with apomorphine, not only reduced the increase in pallidal activity caused by apomorphine but also markedly inhibited pallidal cell activity, often bringing the cells to a complete stop. Since this inhibition was much greater than that produced by haloperidol alone, the results suggest that apomorphine may be causing a subtle change in the dopaminergic modulation of globus pallidus transmission that is greatly enhancing the ability of haloperidol to inhibit firing.

Amphetamine caused a much more dramatic and consistent increase in pallidal activity than did apomorphine. Amphetamine's effects were attenuated by reserpine and alpha-methyl-para-tyrosine pretreatment and blocked by haloperidol, suggesting that they were related to amphetamine's ability to release dopamine. We are currently examining the relative effects of d- and l-amphetamine to further investigate the roles of dopamine and norepinephrine systems in mediating amphetamine's pallidal stimulation. It is interesting that this drug, which acts by releasing dopamine has a much more profound effect on pallidal activity than does the dopamine agonist, apomorphine. Amphetamine's effects also differ from those of apomorphine's in that amphetamine pretreatment does not potentiate haloperidol's ability to inhibit pallidal activity. These results seem to suggest that stimulating dopamine receptors by different mechanisms may produce different postsynaptic effects. Whether this is due to the agonist's ability to differentially stimulate or partially block different types of dopamine receptors or to reach receptors at pre- or postsynaptic sites different from those exposed to amphetamine-released dopamine remains to be determined.

The effects of systemic administration of carbachol were examined in animals pretreated with atropine methyl nitrate, a peripherally active anticholinergic agent. Three quarters of the cells recorded showed dose-related increases in activity after carbachol administration. Muscimol, administered in logarithmically increasing doses, caused a dose-dependent inhibition in the firing of all pallidal cells recorded, when cells in the pars reticulata of the substantia nigra were monitored during systemic administration of this GABA agonist. Picrotoxin, administered after muscimol, was able to increase neuronal firing to baseline levels and above.

There is some correspondence between the effects of these drugs on pallidal activity and their effects on behavior; drugs which decrease movement and cause catalepsy, such as muscimol and haloperidol, decreased tonic unit activity in the locally anesthetized, paralyzed rats, while drugs which cause hyperactivity and stereotypy, like apomorphine and amphetamine, increased tonic pallidal activity. These observations demonstrate that there are populations of neurons in the rat globus pallidus which appear to be sensitive to alterations in dopaminergic, cholinergic and GABAergic transmission. Whether these changes are due to direct effects of the drugs on the neurons themselves, or are the results of changes in the activity of neuronal inputs to the globus pallidus remain to be determined.

(5) Regulation of GABA Synthesis

Previous studies in this Unit have suggested that the activity of glutamic acid decarboxylase (GAD), the enzyme which synthesizes GABA, may be regulated by factors controlling the binding of pyridoxal-5'-phosphate to the apoenzyme. Our attempts to investigate how holoGAD formation changes in vivo when GABA synthesis is increased or decreased have pointed out some of the difficulties in accurately determining endogenous levels of holoGAD. Postmortem changes and alterations of endogenous factors affecting the binding of pyridoxal-5'-phosphate to GAD, such as levels of pyridoxal-5'-phosphate, inorganic phosphate, ATP and undoubtedly other unknowns make estimation of the in vivo levels of active holoGAD difficult. Nevertheless, the fact that holoGAD formation appears to be a process sensitive to many factors, many of which may be affected by the level of impulse flow and metabolic activity in the GABA neurons, suggests that the formation of GAD holoenzyme from pyridoxal-5'-phosphate and apoenzyme may be an important control point in regulation of GABA synthesis. In support of this, we previously demonstrated that Ca^{++} -dependent increases in GAD holoenzyme levels were associated with synaptosomal depolarization. This suggested that increased transmitter release may be associated with an increase in the levels of holoGAD which would lead to an increase in GABA synthesis.

We have concluded our studies of GABA synthesis regulation by investigating the effects of holoGAD levels of two drugs, gamma-butyrolactone and muscimol. These drugs diminish the rate of GABA accumulation following inhibition of GABA catabolism by aminooxyacetic acid administration, an index of GABA turnover. Each of these drugs was administered at a dose which effectively blocks or markedly reduces GABA synthesis, as indicated by the decreased rate of AOAA-induced GABA accumulation after drug treatment. The animals were sacrificed at the appropriate time by the freeze-blowing technique in order to avoid postmortem changes in holoGAD levels. The results obtained with both drugs indicated that the apparent decrease in GABA synthesis caused by these drugs in vivo is not associated with a decrease in the amount of GAD saturated by cofactor. Thus, while an interaction between pyridoxal-5'-phosphate and the apoenzyme may play a role in the regulation of GABA synthesis under some conditions, changes in the amount of holoenzyme were not associated with the apparent drug-induced decreases of GABA synthesis investigated in our current study.

Proposed Course:

(1) Dopamine Autoreceptors and the Striatonigral "Feedback Loop" in Regulation of Dopamine Activity: Effects of Dopamine Agonists

We plan to explore further the ability of the striatal kainic acid injections to attenuate the effects of lisuride on dopaminergic activity. We will determine whether other ergot drugs have effects similar to those of lisuride in this system, investigate the possible role of the norepinephrine system in mediating the attenuation and complete the study of the effects of

amphetamine in the kainic acid lesioned rat. If we find that the effects of amphetamine on dopamine activity are also altered by contralateral intrastriatal kainic acid injections, consideration of amphetamine and lisuride's common properties might provide additional insight into the mechanisms involved in regulation of dopamine function.

(2) The Role of the GABA and Effects of GABAmimetics in the Substantia Nigra and A10 Dopamine Region

We plan to extend our studies of GABA supersensitivity in the substantia nigra by examining the response of the pars reticulata cells to systemic muscimol in animals held for different lengths of time after striatal kainic acid injections. Although we saw no evidence of a supersensitive response to systemic muscimol in rats 3 weeks after lesion of the striatonigral pathway, we are interested in determining whether the supersensitivity we saw with iontophoretic GABA at this time point would be evident in the systemic studies at a later time.

In view of the reported links between the benzodiazepine and GABA receptors in the CNS, we are currently undertaking a series of studies designed to probe the possibility that benzodiazepines may modify the effects of GABA in the pars reticulata of the substantia nigra. We hope to develop a system in which we can study the efficacy of postulated endogenous "benzodiazepine"-type substances, and plan to continue, in the coming year, to probe the role of the GABA system in the substantia nigra.

(3) The Role of Cholecystokinin (CCK) in Dopamine Function

If we are able to obtain a sufficient quantity of good cholecystokinin we may repeat our initial studies with this peptide and investigate further the interaction between this peptide and the dopaminergic system.

(4) Effects of Neurotransmitter Agonists and Antagonists on the Activity of Globus Pallidus Neurons

We plan to continue our studies on the globus pallidus by comparing the effects of amphetamine and apomorphine with those of L-dopa and the ergot-related dopamine agonists. We also hope to examine the effects of noradrenergic and serotonergic agonists in order to better understand the effects of drugs which interact with several transmitter systems such as amphetamine and the ergot derivatives. Eventually we hope to gain more insight into the mechanisms behind the clinical effects of the dopamine agonists by examining the chronic effects of these agents in normal rats and in "parkinsonian-like" rats with 6-hydroxydopamine-induced lesion of the dopamine pathway.

(5) Regulation of GABA Synthesis

At this time we do not plan additional studies on the regulation of GABA synthesis.

Publications:

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ANNUAL REPORT

October 1, 1979 through September 30, 1980

Neuroimmunology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report
October 1, 1979 to September 30, 1980
Neuroimmunology Branch
National Institute of Neurological and
Communicative Disorders and Stroke

Dale E. McFarlin, M.D., Chief

Research in the Neuroimmunology Branch (NIB) is directed at assessment of immune mechanisms in diseases of the nervous system. There are two facets of this work. The first is the study of experimental models of neurological disease, particularly ones associated with infection or autoimmunity. The second is the application of experimental approaches defined in animal and in vitro systems to the investigation of human diseases.

Although many neurological diseases are believed to be virus induced the underlying mechanisms are largely unestablished. A major emphasis in the NIB is to examine the parameters of virus host interaction in animal models of virus related CNS disease. These studies are being conducted in mice because this species is well defined genetically and immunologically. With measles virus several types of infection have been produced. Inoculation of weanling Balb/c mice with the murine-adapted HNT strain of virus produces an acute encephalopathy leading to death. Inoculation of mice with a different genetic background (SJL) or passive transfer of antimeasles antibody into Balb/c mice after infection produces a much different clinical and pathological process. In both types of experiments, only a small number of the animals become acutely ill, while a significant number develop a subacute disease. In contrast to the acute disease, the subacute disease characteristically is associated with perivascular inflammatory response in the CNS. Virus antigen is present in abundant quantities. These models are being used to identify virus and host factors associated with chronic disease. Elucidation of the mechanisms responsible for the experimental diseases should provide insight into the investigation of human diseases.

A major parameter being studied is the antibody response to the measles virus. Parallel studies are being conducted in animals with experimental disease and patients. Animals hyperimmunized with measles, produce antibody directed at each of the six polypeptide components of the virus. This is also true of patients recovering from acute measles infection; however, in this control population, the immune response against individual components of the virus varies. For example, the antibody response to matrix protein is intense in some individuals but barely detectable in others. This was also true of the antibody response against the phosphoprotein and the fusion protein. From these observations it has been concluded that the antigenic properties of the measles polypeptides are not uniform. This could be the consequence of immunochemical properties of the antigens or of host factors or of some

combination. These findings in normal individuals provide background for the interpretation of our findings in Subacute Sclerosing Panencephalitis (SSPE). Although antibody to the six major peptides of measles has been demonstrated in the serum and CSF of patients with this disease, there is variation in the magnitude of the humoral response to individual polypeptides. The amount of antibody to the matrix protein in most patients is relatively low. This is also true of antibody to the phosphoprotein in some patients. Because these findings are similar to those obtained in normal individuals, it seems unlikely that SSPE results from infection by a strain of virus in which a mutation has occurred in the portion of genome coding for the matrix protein. Instead the data favor reduced synthesis of one or more virus proteins by the host.

Similar studies have been directed at the characterization of the cellular immune response to measles and other viruses in man. Lymphocyte proliferation to measles is significantly less than the response to other viral agents in both patients and controls. Studies of purified populations of immune cells demonstrated that T-cells respond to measles, mumps and vaccinia, while B-cells respond to mumps but not to the other viruses. Further analysis of the immune response has been conducted using subsets of human T-cells. Those with Fc receptors for IgG (T_H cells) were consistently found to respond to viral antigens. In contrast the cells which lack Fc receptors for IgG (T_{non-H}) do not respond to viruses, but proliferate in the presence of allogenic antigens. The response of T_H cells was not dependent on the presence of antiviral antibody. The response to measles-infected autologous fibroblasts and that obtained with measles-infected allogenic fibroblasts have been compared and do not differ. Hence, HLA restriction is not operative. These observations constitute the first demonstration that a subset of human T cells responds to viral antigens. The findings are consistent with the concept that T_H cells play a significant role in the regulation of the immune response to viral antigens.

In the assessment of the humoral and cellular immune response to measles virus considerable effort has been made to characterize surface antigens associated with this virus. It is clear that the use of monoclonal antibody, produced by cell fusion methodology, will greatly enhance this aspect of our research. Monoclonal antibody against the 78K protein of measles virus, a surface glycoprotein, neutralizes virus infectivity and inhibits hemagglutination by the virus. These observations provide formal proof of the functional role of this virus component. The monoclonal antibody provides a highly specific tool for studying the synthesis and assembly of the major measles glycoprotein in a variety of cells either lytically or persistently infected with the virus. In addition, initial attempts indicate that it will be feasible to purify the measles glycoprotein with the use of monoclonal antibody. This will enable biochemical, immunochemical and biological characterization of this virus antigen.

Another major area in which an animal model is being used to identify immunological mechanisms related to neurological disease is the investigation of Experimental Allergic Encephalomyelitis (EAE). This research is also being conducted in mice. A number of factors including the antigen source, the type of adjuvant, the amount of adjuvant as well as the genetic background are influential in the production of the disease. The reports of others that mice with a H-2^S background are susceptible have been confirmed in our laboratory and in recent months another susceptible strain, the DBA 1 (H-2^q) has been identified. The capacity to form antibody against myelin basic protein varies among different strains and is also related to histocompatibility background. However, there is disassociation between the capacity to form antibody against basic protein and the capacity to develop EAE. It seems likely that different genes regulate these two aspects of the immune response.

Neuropathologic examination of mice with EAE showed perivascular cuffing with mononuclear cells, a prominent polymorphonuclear response and extravasation of fibrin and red blood cells. Some of these may have resulted from the use of B. pertussis in the sensitization process. Primary demyelination was a transient early in the disease. Axon depletion and gliosis occurred as the disease progressed. The pattern of myelin breakdown included features in common with those described in encephalomyelitis due to murine hepatitis virus and multiple sclerosis.

In mice it has been consistently observed that EAE is easier to induce with whole tissue than basic protein. This suggests that basic protein in whole tissue may exist in a more encephalogenic form or that an immune response directed at other components of myelin may contribute. Antibody formation to one of the myelin glycoproteins has been previously demonstrated. Our most recent studies indicate that the glycoprotein composition of the myelin complex is considerably more complicated than previously recognized. Twenty-two different components have been identified in experiments using radioiodinated lectins with three different carbohydrate specificities. Six of these were removed by chloroform methanol extraction and may contain significant amounts of lipid. Some of the other lectin binding proteins may represent disassociated subunits of oligomeric complexes. These components may be associated with the oligodendroglial membrane, the axolemma, or the myelin sheath per se. An immune response directed at any one of these components could lead to demyelination.

The application of studies in experimental animals to clinical studies has, to date, primarily been directed towards Multiple Sclerosis (MS) and SSPE. A major approach in these studies of MS has been to identify patients in whom genetic and immunological parameters could be examined. This has led to an extensive study of monozygotic and dizygotic twins who are either concordant or discordant for MS. To date, 30 sets have been evaluated and are now well defined clinically and immunologically. A high degree of concordance has been observed in both monozygotic and dizygotic twin pairs. Furthermore, in a group of twins

in whom one individual clearly lacks a history of neurological symptoms, careful examination has revealed subtle but definite abnormalities. In addition, in over fifty percent of the clinically-discordant twin sets, the normal member had abnormalities of spinal fluid immunoglobulins; these included either an increase in one of the immunoglobulins or oligoclonal banding patterns. These observations indicate that a subclinical immune reaction may be occurring in such individuals.

Efforts have been directed at the identification of genetically determined factors in MS. In the past, our laboratory has used sera from multiparous wives of male patients to detect B-cell antigens with considerable specificity for the disease. Our initial studies could not distinguish whether these antigens were specified by the sixth chromosome which encodes for the HLA complex, or were specified by genes on other chromosomes. In attempting to elucidate the mechanism possibly operative in MS, this consideration is important because examples of both human experimental diseases in which two or more genes play a role have recently been described. Evaluation of the B-cell antigens with twins who are either discordant or concordant for MS showed that the antigens segregate with the HLA complex and occur in the normal members of both monozygotic and dizygotic discordant twins. Hence, it seems likely that the sera are detecting antigens which are controlled by the sixth chromosome.

The importance of genetically identical individuals for immune studies is illustrated by our ongoing assessment of the cellular immune responses to viruses. During the initial evaluation of the twins, the lymphocyte response to viral antigens was assessed. In some twins having the identical genetic background, considerable differences in the immune response to certain viruses was found. These twin pairs are being investigated in greater detail in order to dissect the mechanisms responsible for such differences. The experimental approach involves mixing of immune cells and subpopulations of immune cells from individuals who have the identical genetic background but who differ in their response to viruses. It is hoped that analyses of these functions in the twin population will provide relevant information about immune regulation in MS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02202-05 NI
PERIOD COVERED October 1, 1979 to September 30, 1980		
TITLE OF PROJECT (80 characters or less) Immunological Studies in Patients with Multiple Sclerosis and Other CNS Diseases		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: OTHER:	D.E. McFarlin H.F. McFarland B. Gran K.W. Rammohan A.C. Williams J.I. Greenstein J.L. Sever R. Eldridge S.A. Houff	Chief Asst. Chief Clinical Assoc. Clinical Assoc. Visiting Assoc. Clinical Assoc. Chief Geneticist Clinical Assoc.
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LAB/BRANCH Neuroimmunology		
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INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 5.0	PROFESSIONAL: 3.0	OTHER: 2.0
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SUMMARY OF WORK (200 words or less - underline keywords) <p> The general aim of this project is to obtain a more precise understanding of multiple immunological and genetic factors possibly related singly or in combination to the pathogenesis of <u>multiple sclerosis</u>. These include: (1) Determination of <u>histocompatibility types</u> in a carefully selected population of MS patients and appropriate controls. (2) Correlation of histocompatibility data with the humoral and cell-mediated immune response to <u>viruses</u>. (3) Identification of new lymphocyte antigens which may show greater correlation with multiple sclerosis than presently identified lymphocyte antigens. (4) Evaluation of <u>cerebrospinal fluid immunoglobulin content and specificity</u>. (5) Evaluation of <u>families with a multiple incidence of multiple sclerosis and examination of affected and nonaffected members of these families with respect to the above</u>. To minimize some of the variables in the disease, identical and nonidentical twins who are either discordant or concordant for MS are being studied. (6) Similar studies are being conducted in patients with <u>SSPE</u>. </p>		

Project Description:

Objective:

A. Studies of Multiple Sclerosis (MS)

The goal of this project is to establish a more precise understanding of genetic and immunological factors in multiple sclerosis. These factors are being studied in clinically well-defined cases of MS. Numerous interrelated questions are being investigated and include:

1. Examination of the histocompatibility make-up in a population of sporadic cases of MS and appropriate control individuals.
2. Identification of lymphocyte antigens occurring with increased frequency in MS and correlation of these antigens with established genetic and immunological parameters.
3. Evaluation of the cellular immune response to a number of antigens including viruses, components of the nervous system and histocompatibility antigens. This also includes investigation of lymphocyte populations and subpopulations responding to these various antigens.
4. Correlation of histocompatibility make-up with the cellular and humoral immune response to various viral antigens.
5. Evaluation of cerebrospinal fluid (CSF) immunoglobulin content.
6. The above investigations are being carried out in families with a multiple incidence of MS and in monozygotic and dizygotic twins concordant or discordant for MS. The segregation of histocompatibility antigens and differences in immune function between affected and nonaffected individuals are being studied in both families and twins.

B. Studies of Subacute Sclerosing Panencephalitis (SSPE)

Because of the established relationship of SSPE to measles, humoral and cell-mediated immunity (CMI) in this disease are being assessed in a few patients.

Methods Employed:

Patient Populations. All patients included in these studies have been evaluated as either inpatients or outpatients on the NIB service at NIH. Each individual receives a complete medical and neurological evaluation with appropriate diagnostic studies. Patients classified as possible for definite MS are included in the studies.

Studies of familial MS involve families with two or more clinically confirmed cases of MS. Twins either concordant or discordant for MS are being admitted to the Clinical Center in order to document the clinical aspects of each case, to perform extensive laboratory evaluation, and to clinically classify each pair of twins. These studies are performed in collaboration with Neurogenetics Section, IDB. SSPE patients who have the characteristic clinical EEG, CSF and serological findings are studied.

Histocompatibility. Histocompatibility testing for HLA-A and HLA-B antigens are being performed under contract by Dr. Paul Terasaki. HLA-D typing for DW2 antigens is done by mixed lymphocyte culture in our laboratory. Sera from multiparous wives or mothers of patients are being employed in cytotoxicity assays to identify antigens on T or B lymphocytes from MS patients.

Humoral Immunity. Conventional assays (CF, HAI and neutralization) for antibody to various viruses including measles, rubella, mumps, and vaccinia are performed on serum and CSF. Antibody to these viruses is also measured in serum and CSF using radioimmunoassay.

Cell-Mediated Immunity. Cell-mediated immunity to viral antigens are studied in patients and controls using a lymphocyte stimulation assay. The CMI response to measles virus is also studied using purified viral antigens and macrophage inhibition assays. The responses of T lymphocytes and B lymphocytes are studied in these assays. These populations are prepared from peripheral blood using immunoabsorbant columns and rosetting methods. T-cell subpopulations are obtained on the basis of their capacity to react with Fc receptors of IgG and are described more fully in project Z01 NS 02205-05 NI.

Cerebrospinal Fluid. IgG, IgM and IgA levels are quantitated in each CSF by radioimmunoassay. CSF is also being examined for the presence of oligoclonal IgG in collaboration with Infectious Diseases Branch. Further, preparative isoelectric focusing is being used to obtain immunoglobulins with restricted charge heterogeneity. The immunoglobulin in each fraction will be quantitated by RIA and antibody specificity studied.

Major Findings:

1. A major emphasis of the clinical research program has been an extensive study of monozygotic and dizygotic twins who are either concordant or discordant for MS. This represents an extension of investigations of familial MS and was undertaken to identify more precisely the importance of genetic and environmental factors in the disease. Thirty sets of twins have been evaluated. On the basis of defined clinical criteria they have been classified as either discordant or concordant. It was possible to reach a definite conclusion in

twenty-four of the thirty. These included twelve monozygotic and twelve dizygotic. The degree of concordance was six of twelve in the monozygotic as opposed to two of twelve in the dizygotic pairs. This observation that concordance is higher in monozygotic twins supports the concept that a genetic factor may play a role in the pathogenesis of the disease. However, the absence of complete concordance in the monozygotic twins emphasizes the importance of other factors.

There are two other important variables: a) Age. The mean age of the monozygotic discordant and the dizygotic discordant were 39 and 40 respectively. Hence, many uninvolved twins are still at risk. b) Spinal fluid findings. In many of the clinically normal individuals, abnormalities of CSF consistent with the disease were found. Although no individual had an abnormal IgG, the IgM was elevated in three. Oligoclonal bands were detected in one of six unaffected monozygotic twins and five of nine unaffected dizygotic twins. Six other unaffected twins had monoclonal bands. In every discordant twin set, the oligoclonal or monoclonal bands were more intense in the affected than in the unaffected member. However, in three sets, the unaffected had oligoclonal bands while the affected twin had a monoclonal band. The CSF abnormalities assume greater significance when viewed in conjunction with the findings in six of thirty twins in whom a definite decision about concordance could not be reached. In three of the six, the proband had definite or probable MS. The other twin had either a history of neurological dysfunction or abnormal clinical findings which did not fulfill the criteria for a clinical diagnosis. Furthermore, all three had at least one abnormality of the CSF. Taken together, the combined clinical and CSF findings indicate that mild or subclinical disease is occurring in a high percentage of patients. This may relate to environmental factors or genetic factors or both. The former are being intensively studied in collaboration with the Neuroepidemiology Section, ODIR. Evaluation of the immunogenetic background has revealed that HLA-B7, HLA-DW2, and HLA-DRW2 are increased in the twin population. Correlation between these antigens and concordance has not been undertaken, because many of the twins are still at the age of risk and have abnormalities of spinal fluid consistent with the disease.

In the past, our laboratory has used sera from multiparous wives of male patients with multiple sclerosis to detect B cell antigens with considerable specificity for the disease. In our initial studies, it was not possible to distinguish whether these antigens are specified by the sixth chromosome which encodes the HLA complex, or are specified by genes or other chromosomes. This consideration is important because examples of both human and experimental diseases in which two or more genes play a role have recently been described. Evaluation of the B cell antigens in twins who are either discordant or concordant show that the antigens segregate with the HLA complex and occur in normal members of both monozygotic and dizygotic discordant twins, hence, it seems likely that the sera are detecting antigens which are controlled by the sixth chromosome and which are not the consequence of the disease.

The cellular immune response to viruses is being assessed in the twins. During the initial evaluation, considerable difference in the immune response to certain viruses was found in some twins having the identical genetic background. These twins are being investigated in greater detail as described in project Z01 NS 02205-05 NI.

2. Findings in SSPE. Using immunoprecipitation technique described in project Z01 NS 02203-05 NI, seven patients have been studied. CSF from these patients contain antibody against all of the six polypeptides of measles virus.

The antibody specificities of the SSPE patients did not differ from that seen in serum from normal individuals after acute measles infection. Of particular interest was that reactivity against the matrix protein was found. In some patients with SSPE the amount of antibody against the matrix protein was relatively reduced. However, this was also true of the serum from some normal individuals convalescing from acute measles infection. One contemporary theory concerning the pathogenesis of SSPE is that a mutation of virus genome coding for the matrix protein occurs and that this protein is not produced. The demonstration of antibody against this protein indicate that it is present and provides the antigenic stimulus.

In all cases of SSPE the CSF IgG is increased, but the levels of IgM and IgA varied. Oligoclonal IgG has been documented in the CSF. Preparative isoelectric focusing has provided a means for obtaining populations of pure CSF immunoglobulins. The specificity of these is being examined; however, thus a fraction of CSF which reacts with only a single measles polypeptide has not been obtained.

Significance to Biomedical Research and the Program of the Institute:

The total effort in this project is directed toward the investigation of human diseases of the nervous system. Contributions from other basic projects within the Branch are applied to the study of clinical problems. The majority of the effort is aimed at understanding mechanisms involved in multiple sclerosis. This is a major health problem which affects young individuals at the prime of life. Over the past years a number of fragmentary bits of evidence, suggesting possible etiologies and factors contributing to pathogenesis have been suggested. The present investigation is aimed at intensive study of a small group of well-characterized patients. In addition, the use of families in which there is more than one case of multiple sclerosis and twins eliminate some of the variables encountered in studying sporadic cases. Although SSPE is a rare disorder, it is important because of the documented association between this disease and measles virus. Defining the parameters of the immune response to measles may not only provide insight to the pathogenesis of SSPE, but in addition this may provide important information about other disorders of unknown etiology.

Proposed Course:

Work on this project will be directed at establishing a more precise understanding of the findings previously described. This work will focus on characterization of lymphocyte subpopulations in patients with multiple sclerosis. Recently a series of monoclonal antibodies directed against human lymphocyte subsets have become available. These will be used to seek differences in lymphocyte subpopulations in patients with acute chronic forms of multiple sclerosis. This approach will be used to analyze lymphocyte subpopulations in the twins, particularly those who show differences in the immune response to viral antigens. The population of twins which has been evaluated will be followed for the development of new symptomatology. In addition efforts will be made to add additional sets of twins to the series. Particular emphasis will be placed on obtaining monozygotic discordant twins beyond the age of 50. A continuing effort will be made to integrate methods and approaches resulting from nonclinical projects into the clinical research.

Publications:

Williams, A., Eldridge, R., McFarland, H., Houff, S., Krebs, H., and McFarlin, D.: An investigation of multiple sclerosis in twins. Neurology 30: 1139-1147, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02203-05 NI																														
PERIOD COVERED October 1, 1979 to September 30, 1980																																
TITLE OF PROJECT (80 characters or less) The Immune Response Against Membrane Antigens																																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">J.F. Poduslo</td> <td style="width: 30%;">Staff Fellow</td> <td style="width: 10%;">NI</td> <td style="width: 10%;">NINCDS</td> </tr> <tr> <td></td> <td>W.J. Bellini</td> <td>Staff Fellow</td> <td>NI</td> <td>NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>D.E. McFarlin</td> <td>Chief</td> <td>NI</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>H.F. McFarland</td> <td>Asst. Chief</td> <td>NI</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>J.M. Gheuens</td> <td>Visiting Assoc.</td> <td>NI</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>C.L. Koski</td> <td>Guest Worker</td> <td>NI</td> <td>NINCDS</td> </tr> </table>			PI:	J.F. Poduslo	Staff Fellow	NI	NINCDS		W.J. Bellini	Staff Fellow	NI	NINCDS	OTHER:	D.E. McFarlin	Chief	NI	NINCDS		H.F. McFarland	Asst. Chief	NI	NINCDS		J.M. Gheuens	Visiting Assoc.	NI	NINCDS		C.L. Koski	Guest Worker	NI	NINCDS
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SUMMARY OF WORK (200 words or less - underline keywords) <p> The goal of this project is to characterize the immune response to components of <u>myelin</u> and of <u>measles virus</u>. This study has focused on the composition of the outer <u>membrane surface</u>. <u>Glycoproteins</u> have been identified in the <u>myelin complex</u> by the binding of <u>lectin</u> to components electrophoretically separated. An immune response against these surface components of myelin may contribute to immunopathology involving the nervous system. The measles membrane antigens are being characterized and assessed for the capacity to react with immune cells and antibody. <u>Monoclonal antibodies</u> against membrane antigen are being prepared and used in these investigations. </p>																																

Project Description:Objectives:

The surface components of the myelin sheath and the oligodendroglial plasma membrane are important in attempting to define alterations which occur in demyelinating disorders, such as multiple sclerosis. Such components may not only play important recognition roles in the process of myelination or myelin maintenance, but in addition may be readily susceptible to immunological damage or even act as specific viral receptors. Consequently, the identification, isolation and immunological characterization of these surface membrane components is one aim of this research.

Measles virus encodes for two polypeptides, the hemagglutinin and fusion proteins, which are inserted into the host cell plasma membrane. These surface components may constitute targets for immune recognition. Moreover, the immune response to these antigens may play a role in susceptibility or resistance to disease. A major objective of this study is to assess the immune response to measles virus components with respect to both humoral and cell-mediated responsiveness. Animals with experimental measles infection or hyperimmunized with inactivated virus are being used to develop methods applicable to the study of normal individuals as well as patients with chronic disease such as subacute sclerosing panencephalitis (SSPE) and multiple sclerosis (MS).

Methods Employed:

A. Membrane myelin

The brains from adult Lewis rats are rapidly removed and used immediately for the purification of myelin according to the standard procedure. Aliquots of the isolated myelin are solubilized at a concentration of approximately 1 mg protein/ml of sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis buffer (w/v) bromophenol blue, and 10% (v/v) glycerol.

In some experiments, the isolated myelin is extracted with chloroform/methanol (2/1, v/v) to concentrate the high molecular weight proteins. This was accomplished by successive extractions (3x) of myelin at a concentration of approximately 0.5 mg of myelin proteins per ml solvent. After each extraction the remaining myelin proteins are concentrated by centrifugation.

A programmable gradient maker is used to prepare linear gradients of polyacrylamide gel in a vertical slab gel system. Samples of solubilized myelin containing approximately 30 μ g protein are applied to an 8 x 25 mm well in sample volumes of 10-100 μ l. Electrophoresis is carried out for

4 hours. The slab gels are fixed in methanol/water/acetic acid (45:45:10, v/v/v) and either used for the lectin binding studies or stained in 0.2% (w/v) Coomassie Blue dissolved in this fixative. Stained gels were destained overnight, dried and photographed both before and after drying. Proteins of known molecular weights are radioiodinated by the chloramine T procedure and used as standards. Appropriate aliquots were electrophoresed to provide adequate detection by autoradiography.

Three lectins, wheat germ agglutinin, concanavalin A, and soybean agglutinin were iodinated under similar conditions in the presence of their respective inhibitory monosaccharides, (N acetyl-D-glucosamine, N acetyl-galactosamine and D-mannose, D-glucose, respectively). Specific activities of the iodinated lectins were determined. Lectin binding to individual proteins is assessed by direct application of the [125 I] lectins to the gradient slab gel after electrophoresis. The gels are sliced longitudinally into lanes which contained the separated proteins used for the lectin binding experiments. The gel slices are placed in humidified plastic dishes and overlaid with the iodinated lectins diluted in the appropriate buffer. After incubating for 18-24 hrs, the slices are washed in buffer, dried and analyzed by autoradiography by varying the exposure times between 4 hrs. and 2 weeks to optimally visualize the bands. In some experiments, non-specific binding is controlled for by incubating and washing the gels in the presence of the inhibitory monosaccharide. Photographs of the gels and autoradiographs are enlarged to 8 x 10 inches, and the relative mobility of each protein measured directly by determining the ratio of the distance migrated by a given protein and the gel length.

B. Measles virus membrane antigens

Plaque-purified Edmonston measles virus is grown in Vero cells to titers exceeding 1×10^7 PFU/ml. Supernatant virus is first concentrated by ultrafiltration and purified by two successive velocity sedimentations in sucrose. During virus replication in Vero cells, the various components of the virus may be specifically radiolabeled with nucleotides, sugars and/or amino acids depending upon the intent of the study.

The ability of the purified virus to evoke an antibody response in mice and rabbits directed against the surface components of measles is assessed by conventional serology, such as hemagglutination inhibition, hemolysin inhibition and neutralization. Spleen cells from mice hyperimmunized with measles virus are being used to prepare monoclonal antibodies against the components of measles virus. Two days after an intravenous boost, spleens are removed and a cell suspension prepared. Cells are fused with 8-azaguanine resistant murine myeloma cell lines using polyethylene glycol. The resulting hybrids are screened for

antibody formation by radioimmunoassay. Those which produce antibody are cloned by limiting dilution or in soft agar. Positive clones are grown in tissue culture to yield quantities of antibody sufficient for our studies. In addition, antibody-producing cells are injected into the peritoneal cavity of syngeneic mice in order to obtain ascites fluid rich in antibody.

The antibody response to the individual polypeptides of the virus is assessed by a solid phase radioimmunoassay performed in polyacrylamide gels and recently, by immunoprecipitation of internally labeled viral polypeptides followed by fluorographic assessment subsequent to polyacrylamide gel electrophoresis.

Non-ionic detergent extraction methods have been successful in removing the major envelope antigens from the measles virion. The internal antigens or core proteins are then separated from the solubilized surface antigens by differential centrifugation. Envelope antigens are further purified by lectin-affinity chromatography which permits the purification of the major glycoprotein and fusion protein. Purity of the envelope and core constituents of the virus is determined by two-dimensional electrophoresis consisting of isoelectric focusing in the first dimension followed by polyacrylamide gel electrophoresis in SDS in the second dimension. Such methods have been valuable for isolating and examining small quantities of radiolabeled measles components.

Major Findings:

A. Myelin Membrane

In order to analyze the molecular composition of myelin it was essential to develop new methodology which would provide reliable molecular weights of individual components over a wide range and secondly to detect glycosylated components which were present in trace amounts. Pore gradient electrophoresis in the presence of SDS produced high resolution of multiple proteins and permits estimation of molecular weights ranging from 10^3 to 10^6 daltons. Several methods were used to estimate molecular weights. A linear relationship between the logarithm of the molecular weight [$\log(MW)$] and the logarithm of the relative mobility [$\log(R)$] was found; however, this relationship only remained linear over 30-fold range of molecular weights. Linearity over a wider molecular weight range was found in the relationship between $\log(MW)$ and logarithm of the gel concentration at the position reached by the macromolecule $\log(O/T)$. A computer program was developed which provides statistical estimation of the molecular weight for an unknown protein together with the standard error and 95% confidence. In MW weight ranges in which the $\log(MW)$ and $\log(R)$ were non-linear least-square-curve-fitting provided satisfactory estimates of molecular weights.

In order to analyze the glycosylated components of myelin, purified myelin was separated by gradient slab gel electrophoresis. Subsequently radioiodinated lectins were applied and the glycoproteins identified by autoradiography. Using iodinated wheat germ agglutinin, soybean agglutinin and concanavalin A, a total of 22 lectin binding components were identified. The properties of these are listed below:

Component	Estimated Molecular Weight ⁺ (10 ³)	Lectin Specificity*		
		WGA	SBA	Con A
1	607.7	+		
2	196.9	++		++
3	175.1	++		+
4	130.0	+++	+	++
5	109.8	+++		+
6	98.3	(+)		
7	86.6	(+)		
8	75.3	+++		
9	61.8	+		
10	52.2	+		+
11	48.8	+++		+
12	45.6	+	+	+
13	40.3			+++
14	37.3			+
15	35.7			+
16	26.1	+++	+	+
17	23.7	+++	(+)	+++
18	21.8			+++
19	20.4			+++
20	19.6		(+)	+++
21	19.1		(+)	
22	17.0		(+)	

* Based on SDS-PGE of protein standards using least-squares linear regression analysis of the relationship $\log(\text{MW})$ vs $\log(^0\text{oT})$

+ WGA : Wheat germ agglutinin
 SBA : Soybean agglutinin
 Con A: Concanavalin A
 +++ : High reactivity w/o CHCl_3 -MeOH extraction
 ++ : Moderate reactivity w extraction
 + : Low reactivity w extraction
 (+) : Low reactivity w/o extraction

Six of these were removed by extraction with chloroform methanol and may contain significant amounts of lipids. Some of the lectin binding proteins may represent subunits of oligomeric complexes. Although these components may be associated with the oligodendroglial membrane, the axolemma, or the myelin sheath, an immune response directed at any one could lead to demyelination.

B. Measles Membrane Antigens

A purification procedure for measles virus has been developed which maintains the infectious nature of the virus as well as the antigenicity of the viral polypeptide components. Between 2 and 3 mg of virus is routinely recovered from 3 to 4 liters of supernatant fluids.

Fluorographic studies using either ^{35}S -methionine, ^3H -leucine and/or ^3H -glucosamine labeled virus indicate that purified virus is composed of 6 major structural polypeptides. To date, only a single glycoprotein has been identified, ie, the HA or hemagglutinin. This has been confirmed using specific lectins in the gel overlay method. The second surface protein, the fusion protein has a molecular weight on reduced SDS gels of 41,000 daltons and presumably is associated under non-reduced conditions with an 18,000 dalton glycopeptide. The core polypeptides include the nucleocapsid, the phosphorylated nucleocapsid associated "P" protein as well as the M or matrix and L or presumptive polymerase polypeptides.

A solid phase RIA performed in polyacrylamide gels and more recently an immune precipitation assay have made possible a study of the anti-measles polypeptide specificities of animal sera as well as patient sera and CSF. The latter assay takes advantage of a cell line persistently infected with measles virus. All measles structural proteins are synthesized by this cell line and are internally labeled with ^{35}S -methionine. Lysates of the labeled cells are made and used as a source of measles antigen. A portion of the lysate is reacted with serum or CSF and immune complexes precipitated with Staph protein A or specific anti-immunoglobulins. Immune precipitates are then dissolved in gel buffer and measles polypeptide specificities assessed following PAGE and fluorography. Rabbit anti-measles sera react with all measles polypeptides in both the gel overlay and immune precipitation assays and give high titers in conventional serologic assays indicating that the purified virus retains immunogenicity.

Comparison of normal adult, convalescent sera and SSPE sera and CSF by both assays indicate that all sera react with measles virus polypeptides. Although these sera vary with respect to the peptides recognized, no apparent differences in the general antibody response has been observed. A defect in the synthesis or stability of the matrix protein of measles in SSPE has been suggested by others. This defect has

been inferred by the absence of antibody to the matrix protein in sera of SSPE. While similar results have been obtained in this laboratory with SSPE sera and CSF, normal adult sera and acute convalescent sera also were observed to have low levels of antibody to measles matrix protein.

One of the polypeptides of measles virus appeared to be cellular actin with respect to isoelectric point and molecular weight determinations. Immunologic reactivity, however, occurred only with infected cell lysates or with viral polypeptides. Further studies indicate that this component is a cleavage product of the nucleocapsid protein and not cellular-actin.

Detergent treatment of intact virions in the presence of high salt (1M KCl) has led to the selective solubilization of the membrane antigens of measles virus. Two proteins have been found to be associated with the envelope: a) the glycosylated HA and b) the F₁, non-glycosylated component of fusion protein. Variable amounts of the matrix protein and cellular actin have also been observed in this fraction. The majority of the latter components partition with the internal antigens or core proteins. Lectin affinity columns have been useful in the isolation of the HA protein of measles. Studies using iodinated membrane components indicate that F₁ component of fusion protein is also bound to the column. There are no data to indicate that this protein is glycosylated; however a complex between F₁ and a smaller glycopeptide (F₂) has been suggested. Preliminary results using the gel overlay method on unreduced measles antigen with iodinated lectins indicate binding in the 60,000 dalton region consistent with the proposed F₁-F₂ complex glycoprotein (F₀ or fusion protein). Monospecific antibody to some measles polypeptides has been obtained by hybridoma technology. Thus far, several hybrids that produce antibody to the hemagglutinin of measles have been cloned. One of these has been rigorously characterized. The antibody reacts exclusively with the HA polypeptide in immune precipitation, inhibits hemagglutination, neutralizes measles infectivity and is IgG₁K. The antibody has been purified from ascites fluids and has been coupled to sepharose using cyanogen bromide. This immune adsorbant is presently being used to purify quantities of the HA protein sufficient to study the cellular immune response of normal individuals as well as those with SSPE and multiple sclerosis.

Significance to Biomedical Research and the Program of the Institute:

Myelin is an important component of the nervous system. Characterization of the molecular components should provide insight into the function of the membrane under normal conditions. Elucidation of surface antigens should provide clues of possible abnormalities in diseases involving peripheral and central myelin. Measles virus is a ubiquitous infectious agent of man, producing in most cases a self-limiting disease. Serious complications can arise including

pneumonia and encephalitis. This paramyxovirus appears capable of establishing a persistent infection which leads to a slowly progressive disease, SSPE and has been implicated in multiple sclerosis. In addition, widespread immunization with live virus is currently practiced nationwide. Little is known about those components of measles virus that invoke a humoral and/or cellular immune response in man. Through examination and comparison of the immune response elicited in normal individuals with that of individuals suffering from measles-induced disease states, certain differences may appear which are important in understanding the mechanisms leading to disease. Emphasis has been placed on the purification and chemical characterization of the membrane antigens due to the accessibility of these components to the immune system and the feasibility for these proteins to alter normal surface topography.

Proposed Course:

Studies of the cellular and humoral immune response to the antigen of measles virus, as well as the components of the myelin membrane, will be expanded in experimental and human diseases. Continued efforts will be made to purify sufficient quantities of the individual membrane components by conventional means as well as hybridoma immunoadsorbents. Chemical characterization of the surface polypeptide will also be pursued with emphasis on the disposition of these components in membranes. Such efforts are paramount to the understanding of the interaction between the immune recognition systems and membrane surfaces.

Publications:

Trudgett, A., Bellini, W.J., Mingioli, E.S., and McFarlin, D.E.: Antibodies to the structural polypeptides of measles virus following acute infection and in SSPE. Clin. Exp. Immunol. 39: 652-656, 1980.

McFarlin, D.E., Bellini, W.J., Mingioli, E.S., Behar, T.N., and Trudgett, A.: Monospecific antibody to the haemagglutinin of measles virus. J. Gen. Virol. 48: 425-429, 1980.

Poduslo, J.F., Harman, J.L., and McFarlin, D.E.: Lectin receptors in central nervous system myelin: Identification of membrane glycoproteins after sodium dodecyl sulfate-pore gradient electrophoresis (SDS-PGE) using radioiodinated lectins. J. Neurochem. 34: 1733-1744, 1980.

Poduslo, J.F., and Rodbard, D.: Molecular weight estimation using sodium dodecyl sulfate-pore gradient electrophoresis (SDS-PGE). Anal. Biochem. 101: 394-406, 1980.

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TITLE OF PROJECT (80 characters or less) Immunologic Mechanisms Operative in Experimental Autoimmune Diseases of the Nervous System																						
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SUMMARY OF WORK (200 words or less - underline keywords) The aim of this project is to identify the relative role of various mechanisms operative in the production of <u>experimental allergic encephalomyelitis</u> , a model of autoimmune disease. The immune response to <u>myelin basic protein</u> is being assessed by measuring antibody and in vitro proliferative responses in various strains of mice. The chemical components responsible for disease production, <u>T-cell proliferation</u> , and reactivity with antibody are being studied. The pathology in affected animals is being characterized. These studies focus on the relationship to the host genetic background.																						

Project Description:

Objective: EAE, a model autoimmune disease of the CNS, bears a number of clinical, histological, and immunological features similar to human demyelinating diseases and to chronic encephalitis. The object of this project is to delineate the mechanisms responsible for the pathogenesis of EAE. Susceptibility to EAE and the immune response to myelin basic protein (BP), the established encephalitogenic antigen in a number of species, is being studied in various strains of mice with different genetic backgrounds in order to elucidate the relationship between histocompatibility background and disease mechanisms.

Methods Employed:

In the past EAE has been studied in rats, guinea pigs, and mice. Current investigations are being performed in the mouse because this species is well characterized immunologically and because other research in the NIB is being conducted in this species (Project Z01 NS 02205-05 NI). A number of inbred strains of mice are challenged with either whole central nervous tissue or BP in various adjuvants. BP from several sources is being employed. Clinical disease, CNS pathology, cell-mediated immunity and antibody formation are being evaluated.

Lymphocyte stimulation (LS), macrophage migration inhibition and cytotoxicity are being used to assess immune function in vitro. Optimal culture conditions have been established for the assays which are performed in conjunction with column separations and transfer experiments described below. Lymphoid cells are studied from different sources including lymph node cells (LNC), spleen cells (SC), and peritoneal exudate cells (PETELS).

Lymphocytes are separated into T-cell and B-cells with nylon wool columns and immunoabsorbant column with anti-IgG coupled to Sephadex. Lymphoid cells are treated with monospecific antisera and complement to deplete certain subpopulations of T-cells and to enrich for others. Lymphoid cells, separated by these methods, are characterized by FA, cytotoxicity, and LS as measured by thymidine incorporation.

LNC, SC and column-purified T-cells from the above sources are evaluated for the ability to transfer EAE into normal syngeneic animals. The encephalitogenetic responses versus dose of transferred cells are determined by clinical and histological grading of the recipients. Anti-BP antibody is measured by solid phase RIA.

Major Findings:

EAE can be induced by the injection of murine spinal cord in complete Freund's adjuvant followed by two boosts of H. pertussis given intravenously 1 and 3 days later. SJL, A.SW, DBA/1, B10.S animals

develop severe clinical disease with associated CNS pathology. A.TH and A.TL mice show mild diseases, primarily manifested by weight loss which is accompanied by low grade histological lesions. Balb/c and P/J are generally resistant but histological lesions are found in an occasional animal. No evidence of disease was seen in AKR and C57BL/10 animals. The disease developed earliest (11-13 days post-inoculation - PI) and was most intense in the SJL strain. It occurred later in DBA/1 and A.SW mice (14-18 days PI) and frequently was non-fatal. The neuropathological findings are essentially the same in all three strains. Perivascular cuffing, though present, was less pronounced than in other species. There was a predominant polymorphonuclear response, and extravasation of fibrin and red cells. Primary demyelination was a transient, early feature of the disease process in mice, but nerve fiber depletion and gliosis occurred as the disease progressed. The observed myelin degradation most commonly involved the ingestion by macrophages of small fragments of dissociated myelin via crypts or infoldings of the cell surface at the bases of which were pinocytotic coated vesicles. A similar pattern of myelin breakdown has been described in mouse hepatitis virus encephalomyelitis and multiple sclerosis.

An important variable in the studies is the source and the dose of the H. pertussis. It is not known whether the adjuvant exerts action on the immune system or by altering the permeability of the nervous system. Only mild disease has been produced by the administration of BP and no difference in the encephalitogenicity of BP from various sources has been identified to date. The observation that BP is less encephalitogenic than whole spinal cord suggests that other antigens may be involved. For example, one of the myelin glycoproteins as discussed in Z01 NS 02203-05 NI. Alternatively determinants may exist on the BP molecule in situ which contribute to the immune response and disease induction, but are destroyed during the purification.

Investigation of the immune response to BP has shown that the presence and magnitude of antibody formation is related to a number of variables including the type of Mycobacteria, the dose of Mycobacteria, the use of pertussis and the strain of mouse. The relationship of this response to the genetic background, and specifically to the H-2 haplotype, was investigated. H-2^K and H-2^A animals were found to be high responders with all types of adjuvants; H-2^S animals were intermediate responders; H-2^D, H-2^Q and H-2^B animals were poor responders, and H-2^D responded poorly with M. butyricum, but were intermediate responders with M. tuberculosis. These observations coupled with those on susceptibility indicate that production of antibody to BP and susceptibility to EAE may be dissociated and under the control of different genes. EAE is believed to be cell-mediated. Studies of the T-cell response to BP in the mouse have been initiated. Proliferate responses can be obtained with PETELS while only minimal responses can be elicited with LNC and SC. Macrophage migration inhibitory factor is produced when lymphoid cells from responder strains are incubated with BP.

Significance to Biomedical Research and the Program of the Institute:

A major function of the immune response is to provide protection against infectious agents. Similar mechanisms can lead to disease through either autoimmunity or immunopathologic reactions. It is becoming increasingly apparent the control and regulation of the immune response is complex and varies greatly. In the present project, experimental animals which can be controlled in regards to age, sex and genetic background. In addition, well-characterized antigens are being used to dissect various parameters of immune regulation which can lead to neurological disease. In addition to producing new information about pathogenesis of experimental disease, this project is providing background for the development of new approaches and techniques to study human diseases as outlined in other projects (Z01 NS 02202-05 NI).

Proposed Course:

Future studies will focus on prevention and modification of EAE and investigation of immunoregulatory factors which influence the immunopathologic process. Our studies to date have surveyed a number of mouse strains; future investigations will focus on selected strains which are high responders, low responders and those which develop EAE. Attempts will be made to adoptively transfer murine EAE with lymphoid cells. In some experiments such cells will be restimulated in vitro in order to enhance the transfer. If the disease can be effectively and reproducibly transferred with immune cells these will be characterized using lymphocyte markers.

Publications:

Raine, C.S., Barnett, L.B., Brown, A., Behar, T., and McFarlin, D.E.: Neuropathology of experimental allergic encephalomyelitis in inbred strains of mice. Lab. Invest. 43: 150-157, 1980.

NOTE: This project was formerly entitled, "Immunologic Mechanisms Operative in Experimental Allergic Encephalomyelitis (EAE) and Myasthenia Gravis (EAMG).

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PI: OTHER:	H.F. McFarland D.E. McFarlin J.I. Greenstein W.J. Bellini K.W. Rammohan R.A. Lazzarini M. Dubois-Dalcq	Asst. Chief, Chief Clinical Assoc. Staff Fellow Clinical Assoc. Section Chief Res. Microbiologist
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SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this study is to examine the <u>host immune response to viruses</u> which can produce either acute or chronic infection of the CNS. These studies will examine the host immune response with relationship to mechanisms of protection as well as disease production. In addition, attention will be directed at the viral immune response to viruses in order to permit identification of <u>disease associated abnormalities</u> .		

Project Description:

Objective: This project is designed to examine the virus-host interaction of various models of virus-induced central nervous system disease. These studies are being focused on the role of several variables thought to be important in disease production. These include the biological properties of the virus, the immune response of the host to the virus and the influence of the genetic background of the host on disease susceptibility. In particular, the roles of these variables in establishing chronic viral infections of the CNS, as well as in control or potentiation of disease are being examined. These investigations are being performed in the mouse since the immunogenetic parameters of the host are well defined and easily manipulated.

In addition this project includes studies of the normal immune response in man to viruses associated with neurological disease. These studies are designed to examine the various components of the normal cellular immune response to these viruses and to establish their functional significance.

Methods Employed:

A. Virus-induced CNS disease

Acute virus-induced disease of the CNS is being studied in animals infected with either mouse adapted measles virus or vesicular stomatitis virus (VSV). In addition, these viral infections are being used to study the mechanisms by which the acute disease can be modified to produce a subacute or chronic infection.

1. Measles virus. The hamster neurotrophic strain of measles virus adopted to the mouse is being used in these studies. This virus produces an acute CNS disease when inoculated IC into susceptible mice. The pathogenesis of this infection is being studied using the techniques of histopathology, immunofluorescence, and electron microscopic examination. Further, mechanisms of persistence are being examined in strains of mice which are less susceptible. Both susceptible and nonsusceptible animals are being examined with respect to their ability to support virus replication, and their ability to respond to the virus immunologically. Immunological studies include the determination of antibody using a radioimmunoassay and evaluation of the cellular immune response using a lymphoproliferative assay on virus infected monolayers. In addition, attempts to establish a cytolytic assay are underway.

2. VSV. Inoculation of animals with wild type VSV produces an acute disease of the CNS resulting in death in 2-3 days post inoculation. The mechanisms by which this acute infection can be modified are being examined in studies using the R₁ mutant of VSV. This mutant is not

temperature sensitive and replicates readily both in vitro and in vivo. However, R₁ produces considerably less tissue damage than does WT VSV. This has been reported to be due to a mutation which eliminates the virus-induced shutdown of host cell protein, an event associated with virus-induced cell damage.

The neuropathological characteristics of the diseases produced by WT and R₁ viruses are being studied using routine histological technique, immunofluorescence, and electron microscopy. The nature of the immune response to these viruses is being carried out using techniques similar to those employed for measles virus. In addition, the growth curves for both viruses is being studied in brain and spinal cord and the R₁ virus is being examined for reversion to WT in vivo.

B. Virus specific immune response

The cellular immune response to measles, mumps and vaccinia viruses is being studied in normal individuals. The reactivity of T cells and T cell subsets is being examined using a lymphoproliferative assay performed on virus infected monolayers. T cells are being separated into the T_γ and T_{nonγ} subsets on the basis of their ability to bind the Fc portion of the IgG. Further, identification of subsets is being attempted using monoclonal antibodies to subpopulations of T cells.

Major Findings:

A. Acute viral-induced disease

Measles virus infection in susceptible Balb/c mice is characterized by a consistent pattern of clinical disease consisting of hyperactivity and death in approximately 10 days following inoculation. Viral antigen has been demonstrated in neurons throughout the brain although there is a preferential localization in the structures of the limbic system. Infectious virus cannot, however, be recovered. Further, little tissue damage is noted by light microscopy and no inflammatory reaction is seen. Electron microscopic examination of these animals in collaboration with the Electron Microscopy Section, IDB, confirms the nonproductive nature of the infection since no assembled virus is found although abundant viral antigen is present in neurons. Of interest is the frequent localization of measles protein in the postsynaptic terminals. This has suggested that a functional impairment of synaptic transmission may be related to the pattern of clinical disease.

It has been further shown tht the acute disease is less frequent in other mouse strains, such as SJL mice following IC inoculation with measles virus. Only a small number of SJL mice die during the acute period. Viral antigen is present although its appearance is delayed by a few days as compared to Balb/c mice. No apparent quantitative difference

in the distribution, or nature of the viral antigen can be demonstrated between these two mouse strains. However, SJL mice have a mean onset in clinical disease significantly later than the Balb/c.

In addition to the differences in the acute mortality, a portion of the SJL mice develop a more subacute disease which occurs 1-3 months after the initial inoculation. Fluorescent antibody staining of brains of animals with this late disease demonstrate persistence of measles virus antigens in the neurons. In distinction to the acute disease which lacks significant histopathological alterations, the late disease is associated with a moderate inflammatory process. Animals with late disease also are found to have substantial levels of antibody to measles virus.

2. VSV

The acute disease produced by WT VSV consists of death in 2-3 days post inoculation. Histological examination shows some necrosis and mild cellular infiltrate. In distinction, mice inoculated with R₁ mutant of VSV became ill 4-6 days after inoculation. Also the onset of disease in R₁ infected animals is manifested by paralysis of the hind limbs in distinction to the general moribund state of animals inoculated with WT VSV. Death follows approximately 7 days post inoculation.

Immunofluorescent staining reveals considerable amounts of viral antigen in the spinal cord of the animals inoculated with R₁. This viral antigen is localized previously to the anterior horn cells associated with the hind limb paralysis. In addition a more substantial cellular infiltrate is seen. However, the disease does not appear to have a significant immunopathological component since it is not substantially modified by immunosuppression.

3. Cellular immune response to viruses in man

The cellular immune response to measles, mumps and vaccinia viruses has been studied using a lymphoproliferative assay. Although a substantial response of PBLs can be demonstrated to mumps and vaccinia viruses, only marginal proliferation can be demonstrated to measles virus. However, each of these viruses is associated with long term immunity and substantial levels of antibody to mumps and measles persist for many years after infection.

Separation of PBL into T or B cell populations indicates that the response to mumps and vaccinia viruses is predominantly in the T cell population, although a significant B cell response has been noted to mumps. Again, only marginal stimulation was obtained to measles with the purified T cell fraction. Experiments using T cells further separated into the T_γ and T_{non}γ fractions have indicated that the major responding cell to mumps and vaccinia viruses is in the T_γ population, a population

associated with suppression in other in vitro assays. In a few individuals found to be capable of responding to measles virus, this response is also restricted to the T_γ fraction.

Significance to Biomedical Research and the Program of the Institute:

The importance of genetic and immunological factors in susceptibility, potentiation or protection in viral infections is not well established. Further, although the properties of the infectious agent are certainly of major importance in determining the effect of infection on the host, the actual virological characteristics important in producing persistent infection in distinction to cell death are largely unknown. The delineation of the normal cellular immune response to viruses associated with neurological disease in man will allow a more precise identification of abnormalities which may be related to specific disease states. It is hoped that the studies outlined in this project will help to identify the host and virological factors involved in the production of chronic neurological diseases, such as Subacute Sclerosing Panencephalitis (SSPE) and possibly Multiple Sclerosis (MS) and Amyotrophic Lateral Sclerosis (ALS).

Proposed Course:

These studies will focus on the various host mechanisms responsible for persistence of virus and modification of acute viral infections using the measles virus and VSV models. Major attention will be given to the role of immunological mechanisms in protection and disease production in these examples of persistent viral infections. Further, studies will be directed at establishing the significance of the T_γ restricted response in man and to establishing the functional roles of the T cell subpopulations in man.

Publications:

McFarland, H.F., Pedone, C.A., Mingioli, E.S., and McFarlin, D.E.: The response of human lymphocyte subpopulations to measles, mumps and vaccinia viral antigens. J. Immunol. 125: 221-225, 1980.

Rammohan, K.W., McFarlin, D.E., and McFarland, H.F.: Chronic murine measles encephalitis. J. Infect. Dis. (in press).

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